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Genetic diversity among and between Rivercane, Arundinaria Gigantea, Canebrakes assessed by Microsatellite Analysis

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By

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Arundinaria gigantea, a North American bamboo that historically grew in vast canebrakes, is now considered a critical component of an endangered ecosystem. Expressing self-incompatibility, restoration efforts must ensure genetic diversity within canebrakes for viable seed production. DNA fingerprinting methods were developed using 20 simple sequence repeat (SSR) markers and two sequence-characterized amplified region (SCAR) markers. Among 18 markers able to amplify rivercane DNA via polymerase chain reaction (PCR), 10 were demonstrated to be polymorphic within rivercane. Markers could distinguish rivercane among and between canebrakes and could discern full-sibling seedlings. The mostly-infertile Mississippi canebrakes (canebrakes) of rivercane was determined to contain 46% genetic diversity within canebrakes and an average of 1.436 effective alleles. In contrast, the fertile North Carolina canebrakes contained 99% genetic diversity within canebrakes and an average of 6.435 effective alleles. Therefore, theoretically, at least seven distinct genotypes were needed for a healthy, viable rivercane brake.

DEDICATION

This work is dedicated to my God, my mother Angela, my love Tina, my grandfather James, my grandmothers Elnora and Mary, my aunt LaSha, my sisters Alex, Janiece and Taeshia, my brother Stanley, and my Father Scott. I wouldn't have been able to do anything without you guys. Thanks for always being there. Also to all my friends and family who had roles along the way, thank you as well. You were just as important.

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CHAPTER I

LITERATURE REVIEW

Arundinaria gigantea

Botany and plant growth

Arundinaria gigantea (Walter) Muhl., also known as rivercane (or river cane), is one of three native bamboo species in North America. Rivercane is a warm temperate climate bamboo that has grown in areas within the United States (U.S.) as far north as Missouri and Virginia, and as far west to Texas and east to the Atlantic Ocean (Marsh 1977). The remaining two native bamboo species have recently been designated as species based on morphological differences (Platt and Brantley 1997, Triplett et al. 2006 and Triplett and Clark 2010, Triplett et al. 2010) and grow in much more restricted areas: *A. tecta* only grows on the coastal plain of southeastern U.S., and *A. appalachiana* only grows in the southern Appalachian Mountains.

Rivercane is a woody, evergreen, perennial grass in the Poaceae family and is in the Arundinarieae tribe within the Bambusoideae subfamily. It is believed to be a tetraploid (2n=4x=48) like all other bamboo species (Triplett et al. 2010). Rivercane asexually propagates itself via culms arising from rhizomes that generally grow within the upper 15 cm of soil (Platt and Brantley 1997). Rhizomes can span great distances under suitable growth conditions with multiple culms arising from each rhizome. Culm growth initiates in early spring and ceases by mid-summer with growth rates noted to

reach 38 mm per 24-hr period, and can reach heights of 8-10 m (Platt and Brantley 1997). After the end of the seasonal growth period, the culms harden and begin to sprout additional foliage, but their height remains fixed after hardening (Judziewicz et al. 1999). Leaves on the culm are produced at the nodes and are made up of a sheath, inner ligule, outer ligule, and blade (Judziewicz et al. 1999). Between the sheath and blade, the leaf narrows abruptly to form a pseudopetiole. At the node, the axillary bud is capable of producing 3-5 branches. Individual culms can live an average of about five to 10 years (Platt and Brantley 1997).

Historical and ecological significance

Arundinaria gigantea canebrakes, rivercane brakes, were found throughout the southeastern U.S. in savannahs, along streams and rivers and other moist damp areas (Stewart 2007). Historically, numerous explorers reported large, dense canebrakes which were described as being "like an ocean", and required extensive manual clearance in order to proceed (Stewart 2007). Although various historical accounts cite rivercane as a hindrance and overwhelming, this species was commonly used as an indicator of fertile soil and nearby water sources. Therefore, canebrakes would be burned or cleared to make way for agricultural purposes such as fields for crops and for urbanization.

Native Americans such as the Cherokee, Choctaw and Chickasaw Nations used rivercane to make mats, floors, walls, and baskets. Baskets of these tribal nations are seen as treasures and valuable native artifacts that are still made by their modern descendants. Rivercane was functional as a raw material to make personal carriers for tools, and to carry and store food, which are also seen as art within and among tribal nations. It was also used to make weapons such as blowguns to hunt small animals (Platt

et al. 2009). Other items and tools, created from rivercane, or swampcane as some Native Americans called it, were spears, drills for boring holes in rock, pipe stems, and musical instruments such as flutes.

Platt et al. (2009), suggested that despite Native American use of rivercane, this minimally impacted its availability and abundance compared to agricultural practices and urbanization. Early European settlers also had practical uses for rivercane. They would allow livestock to graze on the young shoots of *A. gigantea* because of its abundance and its location being synonymous with water (Platt and Brantley 1997). According to a historical review by Platt et al. (2009), rivercane foliage contained more than 18% of protein and other essential minerals such as calcium and phosphorous. Since rivercane was abundant historically and beneficial to livestock health, it is believed that canebrakes were significantly diminished by overgrazing (Stewart 2007).

Rivercane flourishes under moderate flooding, occasional burning and wind disturbance (Platt and Brantley 1997, Gagnon et al. 2007). Even though rivercane can sustain flooding for a short period of time, eventually it will succumb and die. The same being true for fire; *A. gigantea* canebrakes remain stabilized if burned about every 10 years, but annual burnings will permanently purge the plant from the area (Platt and Brantley 1997).

Restoration of rivercane and faunal inventory is critical for the conservation of this sensitive ecosystem. Historically, because of the size and density of the canebrakes, many mammals took shelter and nested within the culms and foliage of rivercane. Growth of *A. gigantea* is still essential and relevant in modern circumstances because of its roles in wildlife habitation, water buffering, and riverbank stabilization capabilities.

The drastic reduction of rivercane over time has caused a sense of urgency to restore canebrakes to such a degree that rivercane is listed as an endangered ecosystem (Noss et al. 1995). *A. gigantea* is still vital to the survival of many species that are either found in very limited numbers, although many other species have become extinct because of the drastic decline in canebrakes throughout the Southeast. Remsen (1986) argued that canebrake destruction was responsible for the disappearance of Bachman's warbler (*Vermivora bachmanii*), five butterfly species, and decreased numbers of swamp rabbit (*Sylvilagus aquaticus*), Louisiana black bear (*Ursus americanus luteolu*) and bison (*Bison bison*) in the southeastern US. Rivercane has also been suggested as a foraging habitat for the American alligator (*Alligator mississippiensis*), a potential seed source for the extinct Carolina parakeet (*Conuropsis carolinesis*), and home to cotton mice (*Peromyscus gossypinus*, Platt et al. 1997, Platt et al. 2013). Rivercane's growth characteristics enable it to act as a riparian buffering system, (Robinson et al. 1996, Schoonover et al. 2005). Riparian buffers can serve as erosion control and sediment deposition areas. Rivercane's three important attributes: (1) fine roots tightly bind soil particles together; (2) fibrous root network increases soil porosity and promotes infiltration of surface runoff; (3) dense vegetation slows the velocity of surface runoff passing through the buffer, spreading it more uniformly over the ground surface (Robinson et al. 1996). Canebrakes can also act as a buffer for ground water in removing sediment due to these attributes especially the tight compaction of soil particles by fibrous roots which causes the velocity of the surface water to slow, allowing sediment to settle (Schoonover et al. 2005). A study by Andrews et al. (2011) observed that restoration of stream banks using forest vegetation and *A. gigantea* significantly

improved water quality by reducing nitrate, chloride, sodium and potassium levels. When dissolved solids, such as these, are found in significant amounts in freshwater, they can cause ecological disturbances such as emitting odors and inducing gastrointestinal distress after ingestion. It is important to prevent nutrients such as nitrates, phosphates and ammonia nitrogen from entering the Gulf of Mexico because they can contribute to "dead zones" (reduced level of oxygen in the water) that can cause harm to the seafood industry, marine and freshwater ecosystems.

These studies highlight the notable properties of rivercane that can positively impact, both ecologically and economically, water quality, stream/river bank stability and endangered species conservation to name a few. Understanding rivercane genetically, including asexual and sexual propagation (discussed below), should aid in these efforts.

Propagation and reproduction

Viability of rivercane brakes is essential to the successful restoration of this species. Several studies have been conducted exploring reproductive mechanisms in bamboo, including *A. gigantea* (Baldwin et al. 2009). Bamboos flower sporadically and gregariously making it hard to perform a thorough study to completely understand reproduction. Rivercane flowers in 30-40 year cycles (Judziewicz et al. 1999); extreme winter temperatures, drought, cutting or burning of culms have been suggested to stimulate flowering (Hughes 1951). However, attempts to induce flowering experimentally have been limited and largely inconclusive, since flowering is most likely regulated by a combination of internal and external factors (Marsh 1977).

After flowering, culms typically die, but Datillo (2005) indicated that adding manure or hardwood mulch could increase chances of restoring a canebrake. Without

intervention, the culms die after flowering and if no seeds are set, the entire canebrake may die off without seedlings to repopulate.

Information regarding rivercane reproduction is scarce, but temperate tropical bamboos are often self-incompatible based on factors involving pollen viability and pollination. However, they can successfully generate progeny via cross-pollination between different genotypes (Koshy and Jee 2001). Studies by Baldwin et al. (2009) reinforced this. A rivercane plant/clone geographically isolated from other rivercane produced >1000 flowers, yet only produced 11 seeds (via self-pollination), with only 3 being viable. This self-incompatibility phenomenon was noted with other geographically isolated rivercane plants. However, manual cross-pollinations among rivercane from different geographic locations (believed to be genetically different) produced 28 seeds (20 germinated, Baldwin et al. 2009).

By understanding how much genetic variation (defined as variation among alleles) or genetic diversity (defined as overall variation within and among canebrakes). is needed to promote fertile stands, rivercane restoration will be more successful. In comparing genotypes between two canebrakes (located in different counties in North Carolina), Mathews et al. (2009) determined that the flowering rivercane within each canebrake were mostly monoclonal (one predominant genotype within each canebrake), and no genotype overlapped between the two distinct canebrakes. Therefore, it was suggested that plants should be collected and incorporated from multiple canebrakes in restoration efforts to ensure genetic diversity for viable seed production. Suyama et al. (2000) reported on the clonal structure of dwarf bamboo (*Sasa senanensis*) in a 10 hectare plot. Of 51 samples taken, 22 different genotypes were identified, and one clone

was demonstrated to have spread 300 m across the plot. This study was an early attempt to characterize the genetic structure of a bamboo population. Results emphasized the mobility of bamboo via rhizomes, so individual canebrakes growing nearby should not automatically be assumed to be genetically distinct/diverse.

Currently, the most understood element of *A. gigantea's* reproduction is vegetative. Rhizomes are usually dug and transplanted. However, cutting rhizomes can cause an embolism, and their preservation can be tedious. Baldwin et al. (2009) described three methods of propagation: seed germination, micropropagation and macropropagation. The study concluded that seed germination was maximized by using the roll towel method and removal of glumes from the seeds could improve germination. Micropropagation yielded shoots, but root growth was inhibited. Macropropagation showed the most promise because mother plants could give rise to as many as 400 rhizome-generated clones and were relatively easy to harvest (Baldwin et al. 2009)

DNA fingerprinting

DNA fingerprinting is a term used to determine and describe genetic identities via DNA profiles, and is used to assist in distinguishing one group or individual from another group or individual. Multiple DNA fingerprinting techniques have been developed with applications that serve purposes such as forensic analysis and determining genetic diversity including phylogeny and phylogeography in a number of organisms. Two review articles on DNA fingerprinting in plants (Agarwal et al. 2008, Nybom et al. 2014) were used to help formulate the descriptions of the molecular marker-based techniques discussed below, and are cited at the end of those specific paragraphs.

Molecular markers, including minisatellites

An early form of DNA fingerprinting in plants relied on simple mutations that destroyed restriction endonuclease recognition sequences, thereby preventing digestion by those specific restriction endonucleases at mutated sites. This was termed restriction fragment length polymorphism (RFLP), a non-polymerase chain reaction technique. RFLP analysis involves hybridizing a labeled DNA probe to a Southern blot of DNA digested with restriction endonucleases, resulting in differential DNA fragment profiles. As indicated above, the DNA profile of one sample that differed from another could be based on the difference in a single nucleotide, or a single nucleotide polymorphism, that caused the DNA to be digested differently. RFLP markers can be polymorphic, inherited co-dominantly, and are advantageous due to their abundance throughout an organism's genome, locus-specificity and overall usefulness for physical mapping. Helentjaris et al. (1986) used RFLP analysis to generate simple genetic linkage maps in *Solanum lycopersicum* (tomato) and *Zea mays* (maize). However, the RFLP technique is not widely used primarily due to tedious and time-consuming steps in the protocol, including the need for large amounts of DNA and use of radioactive- or dye-labeled probes (Agarwal et al. 2008, Nybom et al. 2014).

Two similar types of genetic analysis are amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD). AFLP combines the usefulness of RFLP to differentiate samples with the added specificity and flexibility of polymerase chain reaction (PCR) technology. This reduced the amount of DNA needed for analysis and enabled identification of numerous amplified DNA fragments as well as high resolution with increased PCR stringency. Polymorphisms in

AFLP band patterns map to specific loci, allowing the individuals to be genotyped, or differentiated, based on the alleles they carry. RAPD analysis relies on PCR using primers called "random" which amplify unknown pieces of DNA trying to detect DNA rearrangements/deletions in the amplified DNA, thereby yielding unique banding patterns. No information is needed regarding the plant's DNA sequences but the markers are dominant, which does not enable distinction of heterozygous alleles (Agarwal et al. 2008, Nybom et al. 2014).

Jeffreys et al. (1985) pioneered the field of DNA fingerprinting and DNA profiling in humans (*Homo sapiens*) by demonstrating the utility of hypervariable DNA repeats, named minisatellites, to determine and distinguish multi-allelic variation and genetic identities. They used human minisatellite probes composed of tandemly repeated DNA sequences to hybridize to DNA digested with restriction endonucleases; researchers were able to distinguish individual human samples based on their genetic fingerprints. The first demonstration of minisatellite DNA fingerprinting in plants was in *Oryza sativa* (rice), where researchers used a minisatellite human probe to distinguish rice cultivars (Dallas 1988). Minisatellites are generally tandem repeats of 11-60 bases in length and situated in/near telomeric regions of chromosomes (Gupta et al. 1996).

Molecular markers in bamboo

Taxonomic identification of plants, at times, relies on the inflorescence morphology (Friar and Kochert 1990). However, as described earlier, reproduction in rivercane and other members of the Bambusoideae subfamily is quite variable and can take decades to flower. Identification is further limited if the plant is sterile, which has been noted as a serious problem in Bambusoideae (Wu 1962). So, efforts have been

made to classify bamboo via AFLP and RAPD analyses. Loh et al. (2000) were able to classify and distinguish four genera of bamboo (*Bambusa, Dendroclanus, Gigantochloa* and *Thyrostachys*) via AFLP analysis. Lin et al. (2009) could distinguish between 10 cultivars of *Phyllostachys pubescens* via AFLP analysis. RAPD analysis was used to distinguish 12 bamboo species representing five genera (*Bambusa*, *Cephalostachyum*, *Dendrocalamus*, *Dinocloa*, *Sasa,* Nayak et al. 2003). Thirty 10-base random primers were tested; 10 yielded amplified products and displayed polymorphisms that enabled species to be distinguished from one another. Since the RAPD procedure can be less reproducible than other fingerprinting procedures, Das et al. (2007) used RAPD analysis to identify random primers that yielded strong bands, then sequenced the amplified DNA to design better, more specific primers; these primers can also be called sequence-characterized amplified region primers (SCARs). The two primers developed, OPA-08 and PW-02 could be successfully used to amplify DNA in 15 different bamboo species in four genera (*Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Pseudobasmbusa,* Das et al. 2005, Das et al. 2007). Additional examples of bamboo genetic analyses via AFLP and RAPD are included in Singh et al. (2013, a review).

An emphasis has been made on classifying *Arundinaria* species via AFLP analysis. Chloroplast DNA was used to determine relatedness of species within *Arundinaria* and between other temperate bamboo species (Triplett and Clark 2010, Triplett et al. 2010). *A. gigantea* could be distinguished from *A. tecta* (switchcane) and *A. appalachiana* (hillcane). Chloroplast DNA data suggested a relatively high divergence of *A. gigantea* from those two species that were more related to each other (sister species, Triplett and Clark 2010, Triplett et al. 2010). Another study by Burke et

al. (2012) used plastome DNA to study the divergence of *A. gigantea* within Arundinarieae. The study concluded that rivercane diverged between 1.94-3.92 mya (million years ago).

Microsatellites

Microsatellite*s* are short DNA sequences, generally 1-6 bases in length, that are tandemly repeated and are inherited co-dominantly. Found in diverse eukaryotic genomes, they are hyper variable and multi-allelic (Senan et al. 2014). Compared to minisatellites, microsatellites are more randomly located throughout genomes (Gupta et al. 1996). They are detected by PCR, using primers that identify, and amplify, these microsatellite sites within genomes. Called SSRs (simple sequence repeats), these short sequences of repeated (repetitive) DNA are found to be widely dispersed within genomes (Tautz and Renz 1984, Schlotterer and Wiehe 1999). Polymorphisms arise due to differences in the number of these repeats, and are based upon strand slippage of DNA polymerase during replication which promotes either excision or addition of repeated sequences. SSRs have been found in numerous plant species and have become very important molecular tools in plant genetics and plant breeding studies because of their availability, usefulness in high throughput systems, and they can be highly polymorphic (Nybom et al. 2014).

Microsatellites in monocots, including bamboo

Using a rice genotype, Chen et al. (1997) developed 94 SSR markers to distinguish members of four rice populations: doubled haploid (two populations), and those generated from a recombinant inbred and an inter-specific backcross. The SSR

markers represented dinucleotide microsatellite repeats. All primers used to identify the SSRs yielded bands, although 7% were faint (Chen et al. 1997). Cordeiro et al. (2001) developed primers to screen 35 SSRs (mostly trinucleotide repeats) in *Saccharum* spp. (sugarcane) which were obtained from a sugarcane expressed sequence tag (EST) library. Among the primers, 60% yielded PCR products in at least one of five tested sugarcane genotypes; among those, 81% were determined to be polymorphic in at least two of the genotypes.

Since microsatellites are found in numerous plant species, even if none have been identified within the plant species of interest, primer sequences from related species could be tested for use in genetic analysis. Marulanda et al. (2007) used 48 rice and 10 sugarcane SSRs to study the genetic diversity among 55 accession of *Guadua* spp. (bamboo in Bambusoideae). Twenty-seven rice and all sugarcane markers could be amplified; four markers (three rice, one sugarcane) generated polymorphisms in all 55 accessions. Nayak and Rout (2005) investigated the use of microsatellites from *Bambusa arundinacea* to begin to assess the genetic diversity of 18 other bamboo species. Of the six microsatellites identified for use, three were determined to be polymorphic and could be of use in future genetic studies. Sharma et al. (2008) used primers for SSR sequences identified in rice (Chen et al. 1997) and sugarcane (Cordeiro et al. 2001) and tested them on 23 bamboo species representing six genera (*Bambusa*, *Dendrocalamus*, *Melocanna*, *Ochlandru*, *Phyllostachys*, *Sasa*). Among the 98 rice SSRs, 44.9% (44) could be amplified in at least one bamboo species; among the 20 sugarcane SSRs, 75% (15) could be repeatedly amplified in at least one species of bamboo making them useful in future genetic analyses. In total, 34 rice SSRs and 8 sugarcane SSRs and could be used to

discern genotypes (polymorphic), because each could detect at least 2 unique fragments (Sharma et al. 2008). Additionally, a study by Chen et al. (2010) verified seven markers that could discriminate between bamboo species using SSRs found on rice chromosomes 7 and 1. A few additional examples are listed in Singh et al. (2013).

Research objectives

Arundinaria gigantea has proven to be an asset, historically, and in our modern environment, but could benefit from restoration and expansion of existing pockets of growth in the U.S. By restoring this ecosystem and habitat, animal species relying on rivercane as a refuge, reproduction site, food source, etc. may remain viable and, perhaps, increase in numbers. Rivercane's morphological characteristics have also been proven useful in stabilizing stream and riverbanks throughout the region, and acts as a natural filtration system. An emphasis on basic research, including determinants of successful sexual and asexual propagation, will enhance the depth and breadth of restoration efforts. Since rivercane is a cross-pollinated species, it will be important to determine genetic relatedness and diversity within and between canebrakes to enable successful sexual hybridization. Use SSR microsatellites and SCARs will be used for these genetic analyses/determinations.

1. Determine the usefulness and sensitivities of SSR and SCAR markers in distinguishing genotypes within A. gigantea.

A panel of SSR and SCAR markers, demonstrated to be conserved in Poaceae, will be optimized for use in screening rivercane germplasm. Their use in detecting genetic diversity within and between canebrakes will be assessed. Since self

incompatibility is a concern in canebrakes, identifying markers that are sensitive enough to determine half-siblings is also a goal.

2. Determine genetic diversity within and among rivercane brakes.

Using the genotype data generated via molecular marker screenings, genetic diversity within and among rivercane brakes will be assessed via statistical analyses. Populations from Mississippi and North Carolina will be analyzed.

CHAPTER II

MATERIALS AND METHODS

Plant samples and sampling methodology

Seed and maternal tissue samples were collected from two seed-prolific rivercane brakes in North Carolina (NC): Cherokee (50 samples) and Cullowhee (50 samples) canebrakes. Tissues harvested from individual sites within a state (population) were called canebrakes. Therefore, Cherokee and Cullowhee are canebrakes and North Carolina samples, as a whole, are a single population. In both canebrakes, individual seeds were used for genotyping, and were separated from the maternal tissue (raceme), if it was still attached. For Cherokee, there were 50 individual seeds and 49 samples of corresponding maternal tissue. For Cullowhee, only 33 seeds yielded quality DNA, and those corresponding maternal tissues were also analyzed. The maternal tissue was separated and its DNA isolated away from seed samples to avoid cross-contamination.

Canebrake samples were collected from six seed-lacking rivercane brakes in Mississippi and one seed-producing stand in Bolivar County, MS (Dahomey National Refuge). The canebrakes were: Bók Turkey (10 samples), Cane Trail (6 samples), Culvert (42 samples), Dahomey (18 samples, full and/or half-sibling seedlings), Shuqualak (17 samples) and Stallo (19 samples) (Table 1, Figure 1). Bók Turkey, Culvert, Shuqualak and Stallo, because of their canebrake sizes, were sampled as indicated: East Stallo (10 samples) were collected starting at the southern-most end of the

canebrake moving towards the northern end at approximately 5 meter (m) intervals. West Stallo (9 samples) were also collected starting at the southern-most end and ending towards the northern end at 5 m intervals. These samples, Stallo, were combined to represent a single canebrake. The Bók Turkey and Shuqualak samples were collected within each canebrake from north to south at 5 m intervals. Culvert was sampled at 5 m intervals across the entire canebrake. Duplicates were collected from the Bók Turkey, Shuqualak and Stallo canebrakes to ensure DNA quantity and quality since, when collected, the PCR conditions and marker standardizations were yet to be completed.

In order to determine the genetic diversity of this species within and among canebrakes, which could be key to understanding the limitations of sexual reproduction of local stands, samples of *A. gigantea* from across central and north Mississippi (28 sites) and 2 Tennessee (TN) sites (Moss Island samples) were also collected and analyzed. Table 1 lists all samples and includes name, physical address/geographic area, county, latitude and longitude. The names of the samples originate from either the geographic site sampled or the Native American name for the watershed as some of these samples were obtained from tribal lands, with permission. Figure 1 shows canebrake locations in Mississippi that were sampled and genetically profiled using gel electrophoresis and the ABI 3130xl Genetic Analyzer (Applied Biosystems). These canebrakes varied in distance from a few meters apart to 300 km apart. Our hypothesis regarding sample collection was samples that were farther away would be more likely to be genetically dissimilar to one another as opposed to more closely located samples since rivercane reproduces both clonally (asexually) and sexually. The closest samples tested came from the Dahomey National Refuge and included three canebrakes: Dahomey,

Cane Trail and Culvert (Figure 2). Individual samples listed in Table 1 were used to ensure that conditions were applicable to amplifying all rivercane samples harvested from multiple locations using SSR and SCAR markers. Also, when rare flowering events were observed, those seed samples were harvested and analyzed.

Seed germination and manual cross-pollinations

According to Baldwin et al. (2009), rivercane flowering events across Mississippi, Tennessee, Alabama and North Carolina were observed between 2006 and 2007. While collecting samples at the Dahomey National Refuge, an inflorescence was observed that contained 18 seeds - which were all collected from the same ramet). These samples were half-siblings since they shared the same mother (collected from same ramet), so they were used in a study to test of the sensitivities of chosen markers (Table 1) to distinguish closely related individuals. Even though these samples were included as a single canebrake they were seeds that were then germinated. This should be noted that they are not technically a canebrake but a "known" and used as such in this study. Basically, although these seedling were genetically analyzed as a canebrake in this study, these seeds were the progeny of a canebrake. Since rivercane is suspected of being highly outbred and may contain a self-incompatibility system, it was unlikely that these seed were the result of self-fertilization. Two geographically distant genotypes (Shuqualak and Bók Turkey, Table 1, Figure1) were harvested and transplanted close to one another, and they flowered naturally. During flowering the racemes of Shuqualak were used to pollinate inflorescences of Bók Turkey. Out of 28 viable seeds produced from this cross, 20 germinated (Baldwin et al 2009). Seed germination was performed according to the methods described by Baldwin et al. (2009). Briefly, germination papers were soaked

with water. Seeds were placed on top of two sheets, leaving 5 cm of unused space on the periphery of the paper. A third sheet was placed on top of the seeds. The bottom margin was folded in then rolled to form a cohesive tube called a roll towel. The seeds were placed in a germination chamber at 30°C (Baldwin 2009 et al.). Progeny (seedling) tissues were analyzed in this study.

Plant tissues for DNA isolation and purification

DNA from the canebrake samples (six large canebrakes) was isolated from young fresh shoots and nodal sections of mature culms. Through preliminary trials by gel electrophoresis quantification, these tissues seemed to yield the most DNA and of higher quality versus DNA extracted from older mature leaves. Samples from the surveyed Mississippi canebrake canebrakes were isolated using leaf and meristem tissue. Samples from North Carolina canebrakes were isolated using individual seeds and both maternal leaf and stem tissue. Seeds from seed-prolific canebrakes were separated from the maternal tissue manually then each underwent DNA isolation separately to avoid cross-contamination. Isolation of DNA from rivercane samples was performed using the ChargeSwitch Forensic DNA Purification Kit (Invitrogen) following the manufacturer's protocol.

ChargeSwitch Forensic DNA Purification Kit (Invitrogen) DNA isolation protocol

The procedure listed below was included with the kit for genomic DNA isolation and purification, and was followed without modification; all solutions were provided by the manufacturer. Plant tissues described above (unweighed) were extracted with sterile scalpel blades to avoid contamination. Processed plant tissue (cut into small pieces, as

directed) was added to a 1.5 ml centrifuge tube (Eppendorf) containing 1 ml of Lysis Buffer and 10 µl of Proteinase K, then incubated at 55°C for 1 h, vortexing every 10 min. Supernatant was added to 200 µl of Purification Buffer plus 15 µl magnetic bead solution, incubated at room temp. 3-5 min, then placed in the MagnaRack (Invitrogen; identified as "rack" below) for 3-5 min. While still in the rack, liquid was removed and discarded, leaving the magnetic beads and newly-charged DNA. Out of the rack, 500 µl Washing Buffer was added, the tube placed back in the rack for 3-5 min, and the wash step repeated. Outside the rack, 50 µl of Elution Buffer (pH >8.0) was added to the DNA/magnetic bead mixture to release DNA from the beads then placed back in the rack to capture the beads, leaving only the DNA in solution; this DNA solution was transferred to a fresh 1.5 ml centrifuge tub

DNA quantification/NanoDrop

DNA samples prepared by the ChargeSwitch Kit were quantified using a NanoDrop 1000™ Spectrometer (ThermoFisher Scientific). This equipment provides DNA quantity and quality values which were used to dilute the DNA to between 0.1 - 2 ng/µl, depending on the sample. Different tissues of the plant (meristem, mature leaves, stems, etc.) were initially processed and analyzed to determine relative DNA quality and quantity based on tissue origin. DNA quantities ranged from 1 ng/µl to144ng/µl. Noted variations of DNA included less quantities of DNA recovered from meristem processed tissues (yielded higher quality DNA) and more DNA from processed mature leaf tissue (yielded lower quality DNA).

PCR

PCR was performed using genomic DNA diluted to 0.1 - 2 ng/µl. Based on prior literature of the marker's origin and standardized using *A. gigantea* samples, 3 - 5 µl of diluted DNA was added to each reaction well containing the master mix. PCR amplifications were performed in a total reaction volume of 10 µl with the master mix containing 1 µl of Gold Taq 10X Buffer, 4% DMSO (buffer), 1 µl (25 mM) $MgCl_2$, 0.3 µl (10 mM) DNTPs, and 0.2 - 0.6 µM/µl primers. PCR were performed on a PE 9600 (Perkin-Elmer) thermocycler using ramping cycling conditions optimized for each set of primers (Table 2). Cycle settings were as follows: 11 min at 95°C, 1 min at 96°C, 30 sec at 94°C, followed by 10 cycles of 30 sec at 59°C, and 60 sec at 70°C followed by 30 sec at 90°C, then 25 cycles of 30 sec at 55°C/57°C/C/60°C/62°C/64°C (as per annealing temperature after optimization), and 60 seconds at 70°C, lastly, 30 min at 60°C.

Primer optimization

Twenty-two microsatellite markers (20 SSR, 2 SCAR, Table 2) were originally selected based on current literature of three genera (Chen 1997, Cordeiro et al. 2001, Das et al. 2007) which showed potential for transferability into bamboo specifically *A. gigantea*. Markers were then tested and optimized based on number of bands obtained, reproducibility and clarity (Table 3). The amount of genomic DNA used in the initial PCR reactions were based on the original literature in which the primer was derived (Chen 1997, Cordeiro et al. 2001, Das et al. 2007), then optimized comparing five concentrations (0.15, 0.2, 0.4, 0.5 or 0.6 µM). Samples were run at different primer concentrations until an acceptable (clearly visible and reproducible) products were

observed. During the optimization of markers, we would identify how many bands were present within a sample (monomorphic or polymorphic) and band size(s).

Annealing temperature optimization

During the optimization process, most markers were tested at the initial annealing temperature reported for the markers in the literature, then optimized using a range from 55°C to 64° C. At least three samples of Dahomey, a ladder (with dye) and a negative control were analyzed in initial screenings using all 22 markers (20 SSR, 2 SCAR, Table 2) at each of five primer annealing temperatures (55°, 57°, 60°, 62° and 64°C). Samples were then screened using different primer concentrations (0.15 µM - 0.6 µM) until an acceptable (clearly visible and reproducible) product was observed. During optimization of markers, we also identified how many bands were present within a sample - if they were monomorphic or polymorphic, and whether there were any distinct bands that could be isolated and used to develop *A. gigantea*-specific markers. In Table 3, some distinct bands were identified for future isolation; for example, RM30 produced a distinct band in Dahomey rivercane samples at around 350 bp. It must be noted that data presented in Table 3 were recorded during the trial phase of primer selection, and mostly Dahomey samples were used for selection purposes as they were abundant and among the first DNA samples isolated. As presented in the results section, other samples created distinctive bands across the Mississippi population with the use of these different markers. The final optimized PCR reaction parameters per marker (first column in Table 3) were deemed satisfactory if results were clear and reproducible.

Genotyping using an ABI 3130xl genetic analyzer

PCR assays using fluorescently-labeled primers were performed using diluted genomic DNA at 0.1-1 ng/µl. In Table 2, markers listed in bold were fluorescently labeled on the forward sequence. Fluorescent primers were purchased from IDT (Integrated DNA Technologies, Coralville, IA) at 100 µM. The labeled dye used for this study was 6-FAM (blue) which was compatible with the ABI 3130xl Genetic Analzyer (Applied Biosystems). The samples were then prepared for fragment analysis by adding a solution of 4.35 µl of formamide, 0.15 µl of GeneScan 500-LIZ (Applied Biosystems) size standard, and 1 µl of post-PCR product to wells of a 96-well plate. Separation and detection of amplified fragments were performed using the ABI 3130xl; it utilizes the sensitivity and accuracy of capillary electrophoresis. Using this instrument, sample DNA fragments are attracted through an ABI "performer optimized polymer" towards a platinum cathode at the end of the capillary, and encompasses the same concept of traditional gel electrophoresis but allows for more output in less time than other traditional methods. Data were analyzed with the Gene Mapper (Applied Biosystems) version 4.0 software package. Gene Mapper is a genetic data management software system optimized for the ABI 3130xl. It allows in-depth customization of experimental settings, simplified data analysis controls, and the ability to measure fragments and score alleles faster with higher output.

All alleles were detected using marker RM251 and RM259, and confirmed reproducible. This meant that in a 96-well plate, triplicates of each sample were analyzed using the ABI 3130xl. This was in order to verify that the peak/allele detected was not an anomaly but real.

In data analysis, markers were considered codominant, as per Chen et al. (1997). Therefore, if only one allele was detected it meant that the second allele is likely the same size allele, or homogenous at that locus; it was counted as two alleles in GenAlEx 6.1 using its codominant data software. For example, Stallo gave a peak at 311 bp using RM251, and would be recorded as 311, 311. If another allele was present, for example 305 bp, then the data input would be 305 bp and 311 bp. The software also takes all markers and combines the alleles detected to develop a genotype. So, if the homogeneous Stallo sample also yielded a peak at 261 bp using RM259, the resulting genotype would be 261, 261, 311, 311. Specific genotypes were not included in this thesis because only two markers were used and the rule of thumb is at least three markers are needed for accuracy. However, using F-statistics and AMOVA we were able to use the alleles detected to determine the number of "effective alleles" (*Ne*) and a basic genetic variation of canebrakes using RM259 and RM251 data.

Statistics

Wright's F-statistics (1946, 1951, 1965) are used to differentiate population genetic structure (Peakall and Smouse 2006, 2012). These statistics allow the partitioning of genetic diversity within and among populations. GenAlEx version 6.1 (Peakall and Smouse 2006, 2012) was used to perform the statistical procedures for frequency-based genetic analysis within and among populations including allele frequency, heterozygosity, F-statistics and Nei's genetic distance. (Note: Heterozygosity in this study is strictly defined as difference in allele/fragment size in loci tested.) Statistical analyses were based on Wright (1946, 1951, 1965), and Peakall and Smouse (2006, 2012). Formulas and variables used are as follows:

-Observed heterozygosity is the amount of observed heterozygous samples divided by the overall number of alleles (Note: *Hetz* stands for the number of heterozygous individuals.) (H_o): $\frac{\#\ of\ Hetz}{Na}$

-Expected heterozygosity is the expected heterozygosity or genetic variation within a population. (Note: *pi* is the frequency of the *i*th allele.) (H_e): $1\text{-}\Sigma pi^2$

-Number of alleles is determined by direct count. GenAlEx also provides the arithmetic mean across loci (N_a).

-Number of effective alleles (N_e) represents an estimate of the number of equally frequent alleles in an ideal population: $\frac{1}{1-He}$

-Fixation index [per locus (F)]: $\frac{He-Ho}{He}$

-Fixation index (among canebrakes) (F_{ST}) is a measure of the extent of genetic diversity among canebrakes. This statistic ranges from 0.0 which means no differentiation and 1.0 which indicates canebrakes are totally genetically different from each other or do not share alleles. H_T is the expected heterozygosity if all populations or canebrakes were pooled (no subdivision). F_{ST}: $\frac{H_T-\bar{H}_e}{H_T}$

-Nei's genetic distance *(D*) and genetic identity *(I*) were also determined using GenAlEx.

D: -ln(I)

I: $\frac{J_{xy}}{\sqrt{J_x J_y}}$

P_{ix} and p_{iy} are the frequencies of the *ith* allele in populations (*x*) and (*y*). *Jx, Jy*, and *Jxy* are the averages of the summation of all alleles and loci in each subpopulation (*x and y*) and dividing by the number of loci. These average values are then used to calculate Nei's *I* via the equations listed below. See Nei (1972) for a more in depth breakdown of the equations mentioned below.

$$\text{Jxy: } \sum_{i=1}^{k} p_{ix}\ p_{iy} \quad (1)$$

$$\text{Jx: } \sum_{i=1}^{k} {p_{ix}}^2 \quad (2)$$

$$\text{Jy: } \sum_{i=1}^{k} {p_{iy}}^2 \quad (3)$$

-GenAlEx uses a tool called "Analysis of Molecular Variance" to calculate population differentiation based on genotypic variation. Using this analysis, *ΦPT* was estimated which is an analog of the previously discussed F_{ST} and is the estimator of genetic diversity among canebrakes. (Peakall and Smouse 2006, 2012). The difference between the population pairwise (*ΦPT*) and F-statistics was the development of a distance matrix which is recognized and more accurate than the former. A pairwise, individual-by-individual (*N x N*) genetic distance matrix is calculated for codominant data by this genetic distance option. For a single-locus analysis, with *i*-th, *j*-th, *k*-th and *l*-th different alleles, a set of squared distances is defined as d^2 (*ii, ii*) = 0, d^2 (*ij, ij*) = 0, d^2 (*ii, ij*) = 1, d^2(*ij, ik*) = 1, d^2(*ij, kl*) =2, d^2(*ii, jk*) = 3, and d^2(*ii, jj*) = 4. This was the best option for codominant data according to Peakall and Smouse (2006, 2012) as it gives a more accurate picture of the overall genetic diversity of populations. The null hypothesis (*H0*) states there are no differences among canebrakes in regard to molecular variance

(ΦPT =0). A population pairwise ΦPT value > 0 indicates a difference in molecular variation among canebrakes (rejection of null hypothesis); the larger the distance from zero, the greater the non-random mating. For AMOVA: *H0* states there are no genetic difference among canebrakes (F_{ST} = 0 = 0 or ΦPT = 0). F_{ST} >0 or (ΦPT> 0) indicates there are significant genetic differences among canebrakes (rejection of the null hypothesis). In AMOVA, under *H0,* canebrakes can be considered part of a single large random mating genetic population (Peakall and Smouse 2006, 2012). This means that if we carry out multiple theoretical genetic shuffles, 999 in this study, we can form a reliable approximation of the range of values we would expect if the null hypothesis were true (Peakall and Smouse 2006, 2012). To determine whether the observed value is significantly greater than that expected by chance, we compared our observed value against the results of the permutations. The probability (p) is calculated as the "number of values ≥ observed value ÷ (number of permutations + 1)" according to Peakall and Smouse (2006, 2012). Within this study, 1000 permutations (999 permutations + your observed results) were used which would prove to be statistically significant, thereby rejecting the null hypothesis at a probability p of < 0.001. Within GenAlEx, AMOVA procedures followed Excoffier et al. (1992), Huff et al. (1993), Michalakis and Excoffier (1996), and Peakall and Smouse (2006, 2012).

Table 1 Bamboo samples collected and locations

Sample	**Address/County**	**Latitude**	**Longitude**
Ackia	3070 Tom Watson Dr, Saltillo Lee Co.	34.314573	-88.695577
Arundinaria tecta (Apookta Chitto)	Black Jack Rd., Bihhi Áyásha Choctaw Reservation, Neshoba Co.	32.788761	-89.234295
Bók Chulaffi Kapassachi	Hwy 330, Yalobusha Co.	33.984126	-89.747548
Bók Okahoma	BIA Rd. 26 (Red Water Rd.), Okahoma Reservation, Leake Co.	32.771139	-89.555635
Bók Shankolo	Cypress Creek and MS 12, Oktibbeha Co.	33.374121	-88.985659
Bók Turkey	17300 Mississippi 330, Yalobusha Co.	33.984213	-89.732903
Cane Trail	MS 446 at Neblett Rd., Bolivar Co.	33.69775	-90.924871
Chinchahoma	2410 MS Hwy 12, Oktibbeha Co.	33.398027	-88.933272
Choctaw Lake	Choctaw Lake		

Table 1 (Continued)

Chulaffi Kapassachi	Hwy 330, Yalobusha Co.	33.984126	-89.747548
Coffeeville PMC	Thad Cochran Plant Materials Center, Holly Springs National Forest, 1046 Bethany Rd, Yalobusha Co.	33.986849	-89.794353
Coonewah	Coonewah Creek where it crosses Hwy. 45 Alt., Lee Co.	34.129846	-88.701304
Culvert	MS 446 at Neblett Rd., Bolivar Co.	33.704365	-90.909556
Dahomey	Dahomey National Wildlife Refuge, MS 446 at Neblett Rd., Bolivar Co.	33.703985	-90.939834
Edinberg at Pearl	River Road, MB Choctaw Indian land, Leake Co.	32.790125	-89.340333
Gum Branch	Sturgis-Louisville Rd., Winston Co.	33.246429	-89.05479
Hasuk Homma	Gore Springs, Grenada Co.	33.772347	-89.618642
Itomi Ikbi	Plymouth Bluff Rd., Lowndes Co.	33.509578	-88.492576

Table 1 (Continued)

Jim Road 2	1417-1419 BIA Rd. 2211 (Jim Rd.), Bihhi Áyásha (Choctaw Reservation), Neshoba Co.	32.822544	-89.288688
Moss Island 2*	Gravel road off Moss Island Rd., Moss Island State Wildlife Reserve, Finley TN, Dyer Co. TN	35.949308	-89.606487
Moss Island 3*	Moss Island Rd., Moss Island State Wildlife Reserve, Finley TN, Dyer Co. TN	35.958137	-89.630191
Nanih Waiya	MS 393 at the Nanih Waiya mound, Winston Co.	32.923194	-88.949439
Noxapater	12618-13108 Mississippi 15, Winston Co.	32.957942	-89.085706
Oktibbeha	2263-2287 Mississippi 25 Oktibbeha Co.	33.350061	-88.879764
Oktoc	Oktoc Rd., Oktoc, Oktibbeha Co.	33.335676	-88.75782
Pseudosasa japonica (Pseudo Oktoc)	Oktoc Rd. Oktoc Oktibbeha Co.	33.335676	-88.75782
Shuqualak	Shuqualak Creek at Airbase Rd., Noxubee Co.	32.958746	-88.571909

Table 1 (Continued)

Skuna Loosa	County Road 221, Yalobusha Co.	33.88003	-89.627473
Stallo	Hwy. 15, Neshoba Co.	32.910129	-89.094211
Tibbee II N	3122 Mississippi 25, Clay Co.	33.556177	-88.658483
Tillatoba Lake	Hwy. 330, Yalobusha Co.	33.975433	-89.82057
Tusca Animpulli	2234 MS Hwy. 12, Oktibbeha Co.	33.406767	-88.917237
Uski Almo	Sturgis-Maben Rd., Oktibbeha Co.	33.463177	-89.053972
Uski Chitto	BIA Rd. 2214 (Beaver Dam Rd.), Bihhi Áyásha (the main Choctaw Reservation), Neshoba Co.	32.808476	-89.213707

All but two samples were *A. gigantea*, with those samples identified
*Samples from Tennessee

Table 2 Rice, sugarcane and bamboo simple sequence repeat (SSR) markers and sequence-characterized amplified region (SCAR) markers

Primer name from plant species listed	Primer sequence - forward	Primer sequence - reverse	Fragment size (bp) in species listed	Dinucleotide or trinucleotide repeats*
Rice (*Oryza sativa*)				
RM30	GGTTAGGCATCGTCACGG	TCACCTCACCACACGACACG	105	$(AG)_9(GA)_{12}$ $(AG)_9A(AGA)_{12}$**
RM31	GATCACGATCCACTGGAGCT	AAGTCCATTACTCTCCTCCC	140	$(GA)_{15}$
RM34	GAAATGGCAATGTGTGCG	GCCGGAGAACCCTAGCTC	161	$(CT)_{17}(TC)_2$
RM215	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	148	$(CT)_{16}$
RM237	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC	130	$(CT)_{18}$
RM242	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG	225	$(CT)_{26}$
RM247	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	131	$(CT)_{16}$
RM248	TCCTTGTGAAATCTGGTCCC	GTAGCCTAGCATGGTGCATG	102	$(CT)_{25}$

Table 2 (Continued)

RM251	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC	147	$(CT)_{29}$
RM259	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGTGCCATGT	162	$(CT)_{17}$
Sugarcane (*Saccharum* spp.)				
MCSA014E10	CTGTACCCGTCGGTCCTCGC	CAGCGGCACTGTCCATGTGC	112	$(GTG)_5$
MCSA053C10	CGAGCATGGCGAGAAGTCCG	GCAGGGCGAGGCGAGATCAG	152	$(CAG)_5$
MCSA062B06	GTCCGCAAGTCCCGTGCTCT	CGGTGAGGTTGTTCTCCACAGG	161	$(CGG)_8$
MCSA077C02	CAACCACCTCGCTCGATTCG	TGATGAGCCAGCAATCCTCCT	144	$(AGG)_8$
MCSA116D08	CAGTCGCCCCACACGCCGAT	CCATGCTGTCGCCGACCACG	174	$(CCT)_5$
MCSA175G03	CCATCGAGCAATCGAGCTGC	GTTTGACAGGCGGATGTTCTGA	114	$(CGG)_6$
MCSA176C03	CCGTCGAGCTGGACTTCACG	CACCTCCACGTCCCACCGAC	243	$(CGG)_5$
MCSA180E02	CAACGACGCAGGATCGAACC	AGCAGGCACGACTTCCCCAC	162	$(CCG)_5$
MCSA205C07	GCTACCAGCTCTCGGTGCTTC	GCACGGGCTAGAACCTAGAAGG	201	$(CGA)_5$

Table 2 (Continued)

YCS02.047	GCAGAGACAGGCGTCTTCGTACT	GCGTTTCCGACCTGGATACC	154	$(CCG)_5$
Bamboo (*Bambusa* spp.)				
OPA-08	GTGACGTAGGCGAACATGGC	GTGACGTAGGGCATACCTTG	209-1706***	
PW-02	TCGTCGGCGTAGACGGAGAG	TCGTCGGCGTTCGAGCTTAT	345-1464***	

SSR marker sequences obtained from Chen et al. 1997 (rice) and Cordeiro et al. 2001 (sugarcane); RAPD marker sequences obtained from Das et al. 2005 (bamboo)

Markers listed in bold were also used as fluorescent labels (forward sequence labeled)

*Dinucleotide information obtained from Sharma et al. 2008; trinucleotide information from Cordeiro et al. 2001

**One dinucleotide sequence from Chen et al. 1997

***SCAR sizes obtained from Das et al. 2007

Table 3 Primer optimization

Primer	Optimized Anneal Temp./[Primer Conc., µM]	55ºC/ [0.4]	55ºC/ [0.5]	57ºC/ [0.2]	57ºC/ [0.4]	57ºC/ [0.5]	57ºC/ [0.6]	60ºC/ [0.2]	60ºC/ [0.4]	62ºC/ [0.15]	62ºC/ [0.4]	64ºC/ [0.4]
RM30	60°C/[0.4]			-				-	350 bp			
RM31	-	-		-				-	-			
RM34	55°C/[0.4]	7 bands		-				-	-			
RM215	55°C/[0.4]	7 bands		-				-	3 bands			
RM237	55°C/[0.4]	6 bands		-				-	-			

Table 3 (Continued)

RM242	-	-		-				-	-			
RM247	-	-		-				-	-			
RM248	55°C/[0.4]	3 bands		-				-	-			
RM251	60°C/[0.4]							300 bp	2 bands			
RM259	57°C/[0.6]			450 bp			2 bands	-				
MCSA014E10	62°C/[0.4]			6 bands				-	10 bands		5 bands	
MCSA053C10	62°C/[0.4]			7 bands				-		2 bands	4 bands	

Table 3 (Continued)

MCSA062B06	60°C/[0.4]			-				-	1000 bp			
MCSA077C02	57°C/[0.4]			400 bp	6 bands			-				
MCSA116D08	64°C/[0.4]			9 bands				-		10 bands		700 bp
MCSA175G03	60°C/[0.4]			-				-	900 bp			
MCSA176C03	60°C/[0.4]			3 bands				400 bp	7 bands			
MCSA180E02	62°C/[0.4]			7 bands				-		900 bp		
MCSA205C07	-			-				-	-			

Table 3 (Continued)

YCS02.047	60°C/[0.4]			2 bands				150 bp	2 bands			
OPA-08	55°C/[0.5]	5 bands	3 bands	-				-	-			
PW-02	57°C/[0.6]			600 bp			2 bands	-				

Annealing temperature/primer concentration combinations that yielded positive results (DNA bands) are presented; as indicated, three primers could not be optimized

(-) indicates negative results; blank cell indicates parameter combination not tested

Note: these were the early trials of optimization for gel electrophoresis using primarily Dahomey rivercane specimens

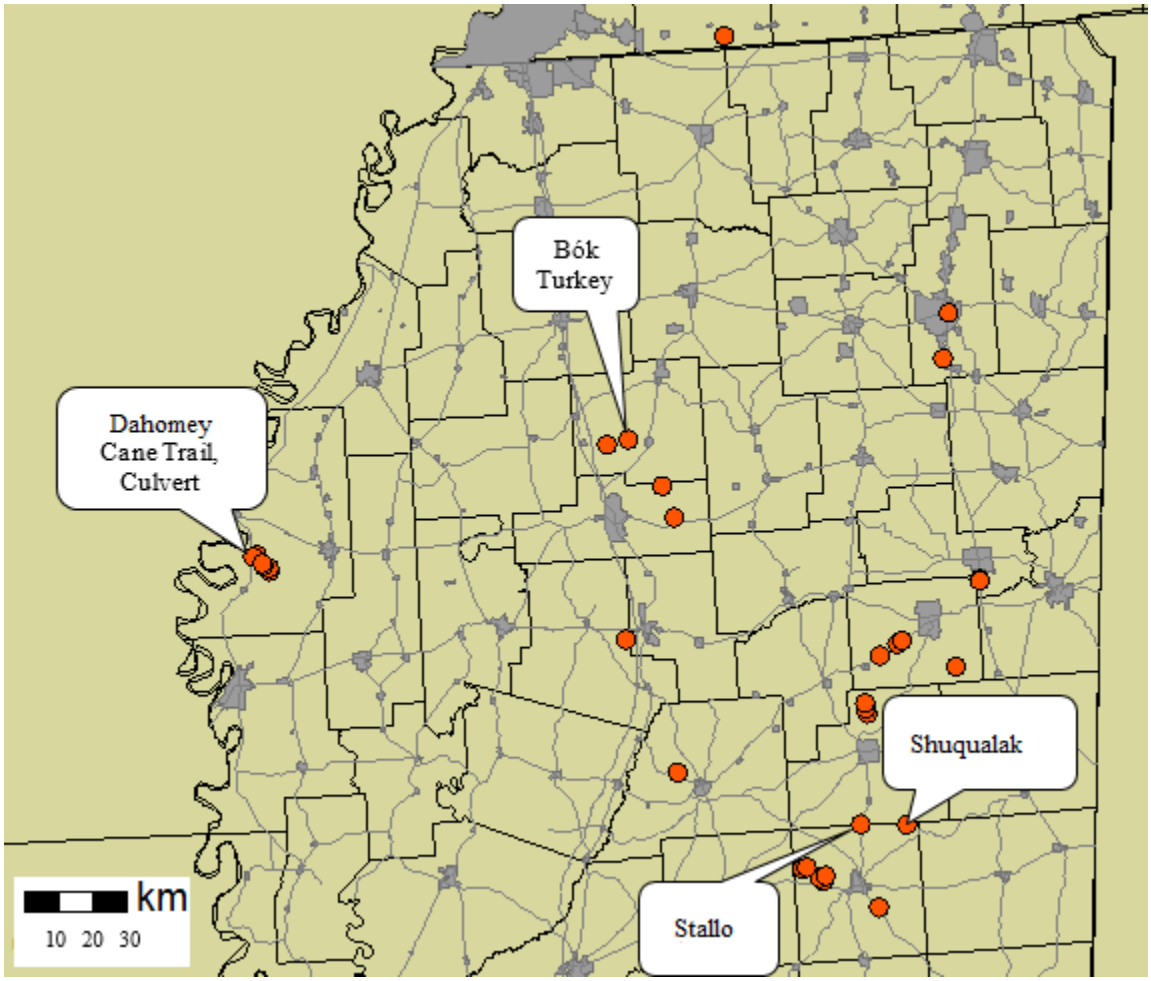

Figure 1 Satellite image of Mississippi rivercane sample and canebrake locations Orange dots indicate where samples were harvested.

Location of the canebrakes where multiple samples were harvested from are indicated (Bók Turkey, Cane Trail, Culvert, Dahomey, Shuqualak and Stallo).

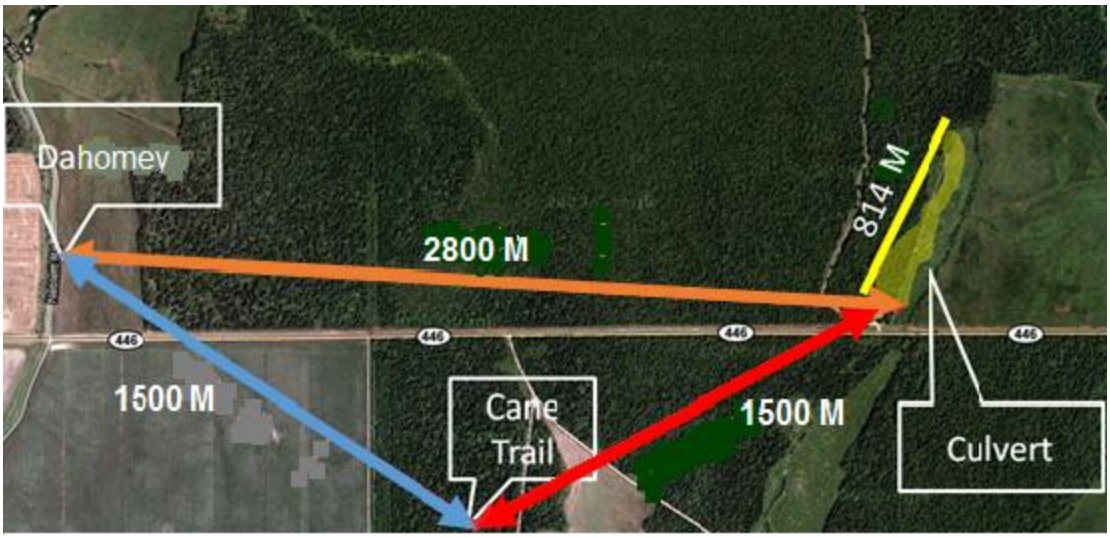

Figure 2 Satellite image of Dahomey, Cane Trail and Culvert canebrakes

Image via Google Earth.

CHAPTER III

RESULTS

Objective I: Determine the usefulness and sensitivities of SSR and RAPD markers in distinguishing genotypes within *A. gigantea*

Primers developed for use in *Oryza sativa* (rice, RM primers; Chen et al. 1997) and *Saccharum* spp. (sugarcane, MCSA and YCS02.047 primers; Cordeiro et al. 2001) based on SSRs and proven to be polymorphic in those species were selected for use (Table 2). They represented SSRs containing di- and tri-nucleotide repeats. Selected rice SSRs represented markers identified on seven of the twelve rice chromosomes, with four showing strong amplification in rice (RM215, RM242, RM247, RM259 primers; Chen et al. 1997). Also included were two sequences identified in *Bambusa* spp. (bamboo) by SCAR analysis (OPA and PW primers; Das et al. 2005).

Before standardization and optimization of the 22 total SSR and SCAR markers, samples of Dahomey and *A. tecta* were quantified using a 1% Reliant Gold (Lonza, Rockland, ME) agarose gel. These samples were used because they were the first samples to be processed in this study. A rivercane DNA standard (analyzed by NanoDrop) was added with either a range between 25-50 ng/µl to the first lane of each gel or an exACTGene (Fisher Scientific, Ontario, Canada) 100 bp DNA ladder. DNA sample concentrations were estimated and diluted between 3 ng/µl and 100 ng/µl. Upon trial and error, we observed that between 3 ng/µl and 5 ng/µl of plant sample DNA was sufficient for band visualization with gel electrophoresis. All primers from Table 3 were

used to determine band visualization. In order to optimize DNA band clarity, gels were run using Reliant 4% NuSieve (Lonza, Rockland, ME) at 110 volts until the 100 bp DNA standard ran completely off the gel. As stated in the materials and methods section, gels were run as samples became available since processing of each sample was tedious. The earliest samples that were processed were from the Dahomey known half-siblings.

The Dahomey siblings were at least half-siblings, and possibly full siblings, because rivercane plants can have multiple paternal sources of pollen and this was not a controlled cross (Baldwin et al. 2009). At least three samples of Dahomey, a ladder (with dye) and a negative control were analyzed in the initial runs with all 22 markers using the following primer annealing temperatures: 55°, 57°, 60°, 62° and 64°C. Different primer concentrations were also compared until an acceptable (clearly visible and reproducible) product was observed (Table 3). During the optimization of markers, we identified how many bands were present within a sample, if they were monomorphic or polymorphic and whether there were any distinct bands (size-wise) that could be isolated and used to develop *A. gigantea*-specific markers. Monomorphic markers did not display differences among bamboo (genera, species, sample site), whereas polymorphic markers did and could be used to discern and identify samples. As indicated in Tables 3 and 4, some distinct bands were identified that could be used for future development and confirmation of primer sets that are rivercane-specific, but was beyond the scope of this research. Most polymorphic markers produced multiple bands, but single bands were correlated with specific samples, so they could be used for such.

MCSA116D08 was the only sugarcane marker to be optimized at an annealing temperature of 64°C but was deemed monomorphic in rivercane. It produced multiple

bands, but all samples yielded the same size/length DNA bands. Markers MCSA205C07, RM31, RM242, and RM247 could not be optimized (Table 3).

Markers polymorphic between bamboo genera and species

Among the 18 markers that were optimized (Table 3), all were found to be polymorphic between bamboo genera and species. Genera and species analyzed in comparison to *A. gigantea* included *A. tecta*, *Bambusa multiplexa*, *Phyllostachys aurea*, *P. nigra*, and *Pseudosasa japonica* (Figures 3-7, Table 5). *Phyllostachys* and *Bambusa multiplexa* were specifically selected because prior literature and morphological features suggested that these species are genetically distant from *A. gigantea* (Burke et al. 2012). A few examples of noted differences are discussed below.

Clear distinctions could be made between *A. tecta* and *A. gigantea* using sugarcane marker MCSA176C03 (Figure 3); *A. tecta* displayed unique bands smaller than 200 bp. A few unique bands could also be discerned among *A. gigantea* samples that represented rivercane brakes from four counties in Mississippi (Table 1). Since the 22 markers used in this study were primarily standardized using *A. gigantea*, DNA from different species did not amplify as well using these markers. For example, samples of *P. nigra* and *P. aurea* analyzed alongside *A. tecta* (lanes 5-7) and Dahomey samples (rivercane; which came from a single location in Bolivar Co., Mississippi) amplified poorly (Figure 4). *P. aurea* yielded bands barely discernable, but *P. nigra* gave a single discernable band that did not seem to be present in most of the rivercane samples (Dahomey). Rice marker RM30 was used to screen many genotypes including *P. nigra, P. aurea, B. multiplexa* and *A. tecta* (Table 5). All four gave different genetic profiles using this marker with bands ranging from 180 to 1150 bp.

Sugarcane marker YCS02.047 proved to be exceptionally polymorphic in discriminating between bamboo genera and species via agarose gel electrophoresis (data not shown). However, this marker could not differentiate rivercane samples via agarose gel electrophoresis, so it was listed as monomorphic for rivercane. However, as discussed later, it proved to be polymorphic when rivercane DNA was analyzed via the ABI 3130xl.

Markers polymorphic among rivercane samples

Among the 18 markers that were polymorphic among bamboo species, 10 were determined to be polymorphic among Mississippi rivercane samples (Table 4). Rivercane PCR products were analyzed initially via agarose gel electrophoresis with the best markers that yielded polymorphisms used in analysis with the ABI 3130xl. Examples of these screenings are presented below.

MCSA180E02 was used to compare DNA harvested from rivercane sites across Mississippi (Figure 5). Samples included rivercane harvested from 12 counties and included the following locations (listed in the order placed on gel in lanes 2-6, 8-20): Dahomey 15, 16, 18, Uski Chitto, Oktoc, Gum Branch, Nanih Waiya, Coonewah, Choctaw Lake, Skuna Loosa, Chinchahoma, Tusca Animpulli, Coffeeville PMC, Oktibbeha, Tibbee II N, Tillatoba Lake, Chulaffi Kapassachi, and Moss Island 3 (Table 1). Among rivercane, the samples displayed varying degrees of variability, although most displayed identical profiles. However, there was enough observed variability for additional analysis using the ABI 3130xl.

Another example of a polymorphic marker is rice RM30 (Table 5). Due to the abundance of DNA samples at the time this marker was analyzed, rivercane samples from

22 locations were compared. Samples also included representatives from three genera and four species, because we were looking for markers that could discern samples between genera and species, and within species. For all samples analyzed (refer to Table 1 for geographic locations), the band sizes obtained via agarose gel electrophoresis ranged from 180 to 1150 base pairs (Table 5). As indicated, polymorphisms were detected among rivercane samples, so this marker was initially screened using the ABI 3130xl Genetic Analyzer.

Arundinaria sp. were compared using two sugarcane markers, MCSA062B06 and MCSA175G03 (Figure 6). Unique *A. tecta* bands compared to *A. gigantea* reinforces results shown in Figures 3 and 4. *A. tecta* is a separate species that displays several unique bands absent within *A. gigantea*. Both markers showed at least one unique band. MCSA175G03 yielded two unique bands with sizes between 200 bp and 130 bp (Figure 6). These unique fragments could, potentially, be sequenced and used as species-specific markers for *A. tecta*. Specifically, among rivercane samples, many polymorphisms were observed using the two markers which presented potential for use with the ABI 3130xl. Samples were also analyzed using rice marker RM30 which yielded some polymorphisms among rivercane samples (Figure 7). Bók Shankolo (lane 7) and Gum Branch (lane 8) did not contain bands present in Uski Chitto (lane 6) and Dahomey (lanes 3-5), and a Dahomey sample appeared to contain a unique 200 bp band (Figure 7).

Markers polymorphic among known siblings

One of the definitive goals of this study was to identify markers that could discriminate among very closely related specimens of rivercane which was suspected in Mississippi canebrakes due to lack of seed production in most canebrakes. Analysis of

Figure 4 showed that within the Dahomey samples, there were some moderate polymorphisms among the samples. This was expected as Dahomey was the only flowering stand in Mississippi that was sampled. In Figure 6, samples run in Lanes 6-8 and 15-17 were collected in the neighboring Mississippi counties of Neshoba, Clay and Winston, respectfully (see Table 1). There were some polymorphisms between these rivercane samples, but they were moderate which could be due to the relatedness of the rivercane samples or just low polymorphisms were exhibited at both loci analyzed (Figure 6). Marker RM30 definitively displayed polymorphisms between siblings analyzed, yielding several discerning bands (Figure 7). This marker displayed discrimination capabilities of both genera and species (Table 5), and could moderately distinguish rivercane samples, including half-siblings using gel electrophoresis (Figure 7).

Overall, among the 10 initial rice markers analyzed, four were polymorphic within *A. gigantea;* among the 10 initial sugarcane markers analyzed, four were polymorphic within *A. gigantea* (Table 4). An additional sugarcane marker (YCS02.047) was also determined to be polymorphic when analyzed using the ABI 3130xl. Both *Bambusa* SCAR markers, PW-02 and OPA-08, were polymorphic in *A. gigantea*. For analyses using the ABI 3130xl (research objective 2), markers needed to display polymorphisms among rivercane samples, and the polymorphic bands needed to be within the size range of 100-400 base pairs. As indicated in Table 2, 11 markers were initially assessed and will be discussed in the next section.

Objective II: Determine genetic diversity within and among rivercane brakes

As discussed previously, a geographically isolated rivercane plant containing 1000 receptive flowers produced 11 seed total (via self-pollination), and only 3 germinated (Baldwin et al. 2009). Studies have been conducted on other bamboos and their reproductive systems, however, none have definitively stated that *Arundinaria gigantea* is outcrossed. Most woody bamboos are highly outcrossed and wind pollinated, so it is inferred that rivercane may also fall into that category. This was reinforced when two geographically distant genotypes (Shuqualak and Bók Turkey, Table 1) were planted next to one another and flowered naturally. During flowering, racemes of the Shuqualak specimen were used to pollinate inflorescences of the Bók Turkey specimen (Baldwin et al. 2009). Out of 28 seed produced from this cross, 20 germinated. These progeny (full siblings) were used to determine sensitivities of 11 markers (Table 2, in bold) that showed potential to discriminate between genotypes in agarose gel-based analyses (objective I).

In order to determine these sensitivities, Bók Turkey, Shuqualak, and two progeny resulting from this manual cross (sibling 1 and sibling 2) were genotyped using the ABI 3130xl using the fluorescently-labeled markers listed in Table 2 (bolded). The markers were assessed for production of peaks (alleles) that were consistent, reproducible (run in triplicate), and within the range of 100-400 base pairs. Using those samples and those parameters, five of the 11 markers showed the greatest potential using the ABI 3130xl.

Based on reproducibility, optimization and polymorphism, the five markers that were more closely analyzed are listed in Table 6. Goals were to construct genotypes and determine if any markers were sensitive enough for sibling (full and/or half) distinction.

Four out of the 5 markers screened were able to determine distinct genotypes of the parents (Table 6). In progeny analyses, only marker RM259 detected one DNA fragment from each parent and these fragments segregated to enable distinction of siblings. These results coincide with Sharma et al. (2008), where the marker RM259 was determined to be one of the most informative and suitable for genetic characterizations in other bamboo.

RM259 was further used in analysis of rivercane canebrakes in Mississippi and North Carolina. RM251 was also included because, although not polymorphic in the study by Sharma et al. (2008) when comparing bamboo species, it was a very useful marker in *A. gigantea*. In fact, RM251 yielded 57 alleles ranging from 100 - 360 bp in our study (Table 7). Using RM259, 37 non-redundant alleles were detected in the North Carolina and Mississippi rivercane samples. It is important to note that only a few alleles were detected in Mississippi samples (two with RM251, six with RM259; Table 7). For example, a sample of Stallo displayed codominant alleles of 311 bp and 311 bp using RM251. That same sample displayed codominant alleles of 261 bp and 261 bp using RM259. Although only one peak was detected in both examples, each was counted as two alleles GenAlEx using codominant data software. These markers were considered codominant (Chen et al. 1997), so if a single allele was detected, the second allele was assumed to be the same size due to codominance. (If a second separate allele was detected using RM251, for example 305 bp, then data input would be 305 bp and 311 bp). The software takes all markers and combines the alleles detected to develop a genotype. So the Stallo genotype was 311, 311, 261, 261 (alleles from both markers RM251, RM259). The genetic distances of all detected alleles were estimated and statistically analyzed by data being input into a distance matrix which was then used to

create an AMOVA table using GenAlEx. See the statistics section of Materials and Methods for formulas used.

Genetic variability among and between Mississippi rivercane brakes

With the destruction of historic canebrakes for agriculture and urbanization, rivercane in Mississippi is suspected to suffer from a reduction of diversity leading to loss of fecudity depression. It is thought that this resulted from the geographic isolation of remaining portions of those canebrakes, and the individuals left behind to rebuild the brake, through vegetative means, contained little genetic diversity. By estimating the genetic variability of the Mississippi rivercane population and comparing it to a healthy seed-producing North Carolina population, we may begin to address reproductive issues not yet understood.

Mississippi canebrakes (Figure 1) were analyzed and compared to determine their relative heterozygosity using *F*-statistics (Peakall and Smouse 2006, 2012; Wright 1945, 1951, 1965). The Culvert and Dahomey canebrakes were the only canebrakes that displayed excess heterozygosity [canebrake fixation index (*F*)] according to the Hardy Weinberg Equilibrium (*F*= -0.081 Dahomey, *F*= -0.035, Culvert) where *F*<0 equals excess heterozygosity (Peakall and Smouse 2006, 2012; Table 8). These canebrakes were also within 1567 meters of one another. The Stallo canebrake displayed the highest *F* (0.586). High canebrake *F* values could indicate severe population fragmentation among canebrakes or highly homogeneous canebrakes (or populations) at that particular locus.

Observed heterozygosity (*Ho*) ranged from 0 to 1, with 1 indicating 100% heterozygous individuals. The highest observed heterozygosity was displayed in the

Shuqualak canebrake at 0.393 (Table 8). According Takezaki and Nei (1996), an average heterozygosity should be between 0.3 and 0.8 to be useful for genetic diversity analysis. The Dahomey and Shuqualak canebrakes were the only groups that met this threshold. Note that samples were collected from the Dahomey stand immediately after a flowering event and was documented as the only stand to produce viable seed during this study. As expected, based Stallo's large fixation index (*F*), it displayed the least observed heterozygosity (0.111; Table 8). The calculated expected heterozygosity (*He*) ranged from 0.181 (Culvert) to 0.324 (Shuqualak) (Table 8). Based on Takezaki and Nei (1996), only Shuqualak values were consistently within the range for analysis of genetic diversity.

The highest number of alleles (*Na*) observed was 2.5 within the Shuqualak canebrake and the lowest was within the Culvert canebrake at 1.5 alleles (Table 8). The number of effective alleles (*Ne*) represents the calculated number of equally frequent alleles in an ideal population. The *Ne* ranged from 1.284 (Culvert) to 1.619 (Shuqualak) and were consistent with the *Na* values in regard to value, overall range, and canebrakes that displayed the lowest versus highest values (Table 8). Both *Na* and *Ne* values indicate that there is little genetic variation within each of the six Mississippi canebrakes. Overall, based on heterozygosity estimates and number of alleles, the two canebrakes that displayed the greatest genetic diversity, albeit limited, were Dahomey and Shuqualak (Table 8).

F_{ST} is an overall measure of the inbreeding coefficient within canebrakes relative to the total population. This statistic provides an estimate of the genetic diversity among canebrakes and, therefore, the genetic divesity within the overall population. With a

scale of 0 to1, the F_{ST} value of 0.523 (Table 8) indicates that the majority of Mississippi canebrakes were genetically different from each other. Another description is that there was a distribution of 52% genetic diversity among Mississippi canebrakes and 48% distributed within said canebrakes. Therefore, this F_{ST} value points to very low variation within Mississippi canebrakes; this was expected due to the noted lack of viable seed production (Baldwin et al. 2009).

F-statistics are useful in genetic analysis, but can be biased if a measured variable is more heavily represented in the sample such as a highly homozygous locus. AMOVA (analysis of molecular variance) uses genetic distance and can provide valid information even if sample size is low due to the use of molecular marker data such as SSRs. The population pairwise value (*ΦPT*) via AMOVA measured the genetic diversity among populations using molecular marker data and is analogous to the F_{ST} (Peakall and Smouse 2006, 2012). *ΦPT* was 0.464 which indicated an inter-canebrake variation of 46% (Table 9). The intra-canebrake variation in Mississippi rivercane was estimated to be 54%. Therefore, there appeared to be only 8% more genetic diversirty within canebrakes than between canebrakes (canebrakes), which was slightly different than F_{ST} data. Looking at both values, it appears that approximately 50% of the noted genetic diversity was found within canebrakes.

The *ΦPT* value was significant (p<0.001), suggesting a significant difference in genetic diversity among Mississippi *A. gigantea* canebrakes (Table 9). Therefore, the null hypothesis of random mating was rejected. This was based on F-statistics (F_{ST}) and AMOVA (*ΦPT*) of these markers and loci (Tables 8, 9). Both values (much greater than zero) indicated significant genetic diversity among canebrakes (versus within). The p

value supported this (Table 9); out of 1000 random mating permutations, only 1 scenario would yield the results observed. So statistically, these results could not be predicted and, therefore, the rivercane canebrakes are not mating randomly.

Genetic variability among and between North Carolina rivercane brakes

The fixation index (*F*) for Cherokee was observed at 0.562 and the *F* of Cullowhee was 0.259 (Table 10), indicating less genetic diversity. These values contradict our theory about greater diversity within North Carolina canebrakes. It must be reiterated that F-statistics are not as reliable as AMOVA when estimating genetic variability according to recommendations from Peakall and Smouse (2006, 2012). Also of note was that, opposed to the six Mississippi canebrakes analyzed, only two NC canebrakes were analyzed.

Observed heterozygosity (*Ho*) among North Carolina canebrakes (seed-producing) were measured at 0.375 and 0.613 (Table 10). These values fell within the range for usefulness in determining genetic diversity (Takezaki and Nei 1996). The observed heterozygosity value noted in the Cherokee canebrake was similar to the *Ho* value for Mississippi canebrake Shuqualak (Tables 8 and 10). The calculated expected heterozygosity (*He*) for both North Carolina canebrakes was 0.833-0.840 (Table 10), greater than two times higher than Mississippi canebrakes Shuqualak and Dahomey (Table 8).

Although Cherokee and Shuqualak canebrakes displayed similar values for *Ho*, the number of alleles (*Na*) observed in Cherokee was 27 versus 2.5 in Shuqualak (Tables 8, 10). Since rivercane displays self-incompatibility (must cross with a different genotype to produce viable seeds), the low *Na* could explain why canebrakes in

Mississippi are not as fertile and, therefore, less seed-bearing than North Carolina canebrakes (high *Na*). Some MS canebrake samples were limited in number, but Culvert had a similar sample size compared to the North Carolina canebrakes and still only displayed 1.5 *Na* (Table 8). This suggests that NC canebrakes are more likely to be healthy seed-producing canebrakes because they are composed of more genetically diverse individuals or genets. Low numbers of alleles (*Na*) due to lack of genetic diversity, could explain why few seeds were collected from stands in Mississippi and among them, the majority were not viable (Baldwin et al. 2009). The number of effective alleles (*Ne*) in the North Carolina canebrakes was 6.424-6.446 (Table 10). Of note is that even with a higher sample mean (*N*) in Cherokee at 64.5, the Cullowhee canebrakes had almost the same number of effective alleles with just a sample mean (*N*) of 18. So even though sample sizes differed, both of these canebrakes displayed the same number of effective alleles. The NC values consistently indicated greater genetic variability existed in these two canebrakes compared to the MS canebrakes (Tables 8, 10). However, when looking at all NC calculated values, the estimates may not have been as reliable as MS estimates since data was only obtained from two canebrakes.

The fixation index (F_{ST}) for all tissues was 0.042 (Table 10) indicating that only 4.2% genetic diversity was noted to be distributed among canebrakes of North Carolina, so 95.8% variation was distributed within canebrakes. Although there were only two canebrakes analyzed in this study, the sample sizes were larger than the Mississippi canebrakes. A F_{ST} of 0.042, a value near zero, indicates random mating within canebrakes.

Due to the abundance of maternal tissues available, they were analyzed separately and F_{ST} calculated (Table 10). This was done because the use of both parent and progeny tissues could bias the results toward a suggestion of inbreeding. Harvested from fertile canebrakes, it was still expected that the overall genetic diversity among the North Carolina canebrakes would remain low. Although slightly higher than the fixation index of all tissues (0.054 versus 0.042; Table 10), it was still near Hardy-Weinberg equilibrium (randomly mating population = 0) as expected.

Peakall and Smouse (2006, 2012) noted that AMOVA was more accurate when discussing and analyzing population genetics because it included a distance matrix in analysis of the detected alleles (see Materials and Methods). AMOVA analysis indicated the inter-canebrake variation in North Carolina rivercane using all tissues was estimated to be 1% ($\Phi PT = 0.010$), significantly lower than intra-canebrake variation (99%; Table 11). This intra-canebrake variation was much greater than the variation estimated within Mississippi canebrakes (54%; Table 9). The ΦPT value of these North Carolina canebrakes was consistent with the calculated F_{ST} (Tables 10, 11); both values were consistent with a population undergoing random mating.

The calculated *p* value for all tissues reaffirms that this observation can be statistically predicted and, therefore, accepts the null hypothesis of a random mating population. From an ecological perspective, this is a sign of a healthy population capable of viable seed production, because even though these two canebrakes are geographically separated, each displayed great polymorphism in the markers analyzed, an indication of genetic diversity. When these canebrakes became geographically isolated the remaining individuals must have been genetically diverse yielding fertile canebrakes. This is in

stark contrast to the Mississippi canebrakes where only 48-54% intra-canebrake genetic diversity was estimated (F_{ST} and ΦPT; Tables 8, 9, respectively), indicating these geographically-isolated, canebrakes re-arose from individuals containing little genetic diversity.

AMOVA analysis of the North Carolina maternal tissue was analyzed to insure the use of the parents and progeny in the "all tissues" samples accurately reflected the amount of genetic diversity present and was not biased toward a conclusion of limited diversity due to harvesting multiple samples from individuals. The AMOVA ΦPT of maternal tissue was -0.020 with a *p*-value of 0.914 (Table 11). According to Peakall and Smouse (2006, 2012), a negative ΦPT indicates non-random sample selection or the intentional selection of genetically diverse individuals. Again, the null hypothesis is accepted indicating there was no significant difference in genetic diversity among canebrakes.

Nei's genetic distance and genetic identity of Mississippi rivercane brakes

F-statistics does not give a good indication of genetic distance because it makes the assumption that all alleles are equidistant. AMOVA, however, analyzes the genetic distance of alleles as well as the allele frequency with the use of a distance matrix. The data presented (Tables 8, 9) suggested that, overall, the Mississippi canebrakes were not very genetically diverse. However, they displayed different degrees of genetic variation, so we wanted to determine if we could tie geographic distance with Nei's genetic distance. Not included in these analyses were the North Carolina canebrakes since there were only two, with one distance between them. Table 12 is a pairwise population matrix

of Nei's genetic distance (*D*) and Nei's genetic identity (*I*) used to further characterize the diversity of Mississippi canebrakes.

Nei's *D* ranged from 0.005 to 1.309 (Table 12). The two canebrakes displaying the smallest genetic distance were Stallo and Shuqualak with Nei's *D* at 0.005. This result correlates with their geographic distance as these canebrakes are within 10 kilometers (km) of each other (Table 1, Figure 1) suggesting that, historically, they might have once been part of the same canebrake. The next smallest Nei's *D* values were clustered among Dahomey, Cane Trail and Culvert canebrakes with values ranging from 0.042 to 0.065 (Table 12). These canebrakes are 1.4-2.8 km apart (Figure 2) and could have once been part of the same canebrake. Members of the two canebrakes manually crossed that yielded 20 viable seeds (Bók Turkey and Shuqualak; Baldwin et al. 2009) were among the most distant canebrakes (approximately 150 km apart), and Nei's *D* (1.157; Table 12) also indicated genetic distance.

Nei's genetic identity (*I*) ranged from 0.270 to 0.995 (Table 12). Similar to the genetic distance data, geographically close canebrakes Stallo and Shuqualak were the most genetically identical at 0.995 while the three canebrakes (Dahomey, Cane Trail and Culvert; Figure 2) were the second most genetically identical with Nei's *I* ranging from 0.937 to 0.959 (Table 12). Referring to the manual cross that yielded viable progeny discussed above, Nei's *I* for Bók Turkey and Shuqualak was 0.314 (Table 12) indicating less genetic similarity. In general, the results from Nei's *D* and Nei's *I* analyses correlate with the geographic distances of these canebrakes using RM251 and RM259, and also reinforces that rivercane canebrakes across Mississippi contain limited genetic diversity as indicated by its lack of genetic distance and genetic identity.

Table 4 Marker polymorphism as assessed by agarose gel electrophoresis

Marker	Annealing Temp.	Primer Conc. (μM)	Polymorphic/Monomorphic*
RM30	60°C	0.4	Polymorphic
RM34	55°C	0.4	Monomorphic
RM215	55°C	0.4	Monomorphic
RM237	55°C	0.4	Monomorphic
RM248	55°C	0.4	Polymorphic
RM251	60°C	0.4	Polymorphic
RM259	57°C	0.6	Polymorphic
MCSA014E10	62°C	0.4	Polymorphic
MCSA053C10	62°C	0.4	Monomorphic
MCSA062B06	60°C	0.4	Polymorphic
MCSA077C02	57°C	0.5	Monomorphic
MCSA116D08	64°C	0.4	Monomorphic
MCSA175G03	60°C	0.4	Monomorphic
MCSA176C03	60°C	0.4	Polymorphic
MCSA180E02	62°C	0.4	Polymorphic
YCS02.047	60°C	0.4	Monomorphic
OPA-08	55°C	0.4	Polymorphic
PW-02	57°C	0.4	Polymorphic

Markers not listed did not amplify during optimization and standardization
*Designation assigned after multiple samples (differed by genus, species, or location) were compared

Table 5 RM30 band scoring table

Plant Sample Source	180 bp	200 bp	215 bp	250 bp	290 bp	310 bp	350 bp	375 bp	380 bp	410 bp	450 bp	500 bp	550 bp	600 bp	640 bp	695 bp	850 bp	1000 bp	1100 bp	1150 bp
Ackia			+	+	+	+				+		+	+	+	+			+		+
Arundinaria tecta (Apookta Chitto)	+		+	+			+					+	+		+		+	+	+	+
Bambusa multiplexa	+				+		+					+		+			+			
Bók Chulaffi Kapassachi	+	+			+			+												
Bók Okahoma			+	+	+	+						+	+	+	+			+		+
Chinchahoma	+	+			+															

Table 5 (Continued)

Choctaw Lake	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+		+	+
Coffeeville PMC	+	+		+	+		+	+	+		+	+			+	+	+		+	+
Coonewah	+	+	+	+	+		+	+	+	+	+	+			+	+	+		+	+
Dahomey 1	+	+			+	+	+	+		+	+	+		+	+			+	+	+
Dahomey 2	+				+		+	+		+		+		+					+	+
Dahomey 3	+	+	+		+							+		+	+		+	+	+	+
Dahomey 4	+		+				+					+			+		+	+	+	+
Dahomey 6					+		+													
Dahomey 7	+		+		+		+		+	+		+		+	+		+	+	+	+
Dahomey 8	+				+		+													

Table 5 (Continued)

Dahomey 9	+	+	+		+	+			+	+	+	+		+	+		+	+	+	+
Dahomey 10	+	+	+		+	+	+		+			+		+	+		+	+	+	+
Dahomey 11	+	+	+	+	+	+						+		+		+	+	+	+	+
Dahomey 12		+	+	+	+	+					+	+		+	+	+	+		+	+
Dahomey 13							+						+	+	+				+	+
Dahomey 14							+						+	+	+				+	+
Dahomey 15	+	+	+	+	+	+	+	+	+		+	+			+	+	+		+	+
Dahomey 16	+	+	+	+	+		+	+	+		+	+			+	+	+		+	+
Dahomey 18	+	+	+	+	+		+	+	+		+	+			+	+	+		+	+
Edinberg at Pearl			+	+	+	+						+	+	+	+			+		+

Table 5 (Continued)

Gum Branch	+	+	+	+	+		+	+	+	+	+	+			+	+	+		+	+
Hasuk Homma	+	+	+	+	+	+					+		+	+	+			+		+
Itomi Ikbi			+	+	+	+						+	+	+	+			+		+
Jim Road 2	+		+	+	+	+				+		+	+	+	+			+		+
Moss Island 3	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+		+	+
Nanih Waiya	+	+		+	+		+	+	+	+	+	+		+	+	+	+	+	+	+
Oktoc	+	+	+	+	+			+	+	+	+	+			+	+	+		+	+
Pseudosasa japonica (Pseudo Oktoc)	+			+																
Skuna Loosa	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+	+

Table 5 (Continued)

Tillatoba Lake	+	+	+	+	+	+		+	+	+	+	+			+	+	+		+	+
Tusca Animpulli	+	+	+	+	+	+	+	+	+		+	+			+	+	+		+	+
Uski Chitto	+	+	+	+	+		+	+	+	+	+	+			+	+	+		+	+

DNA was analyzed via agarose gel electrophoresis. Band sizes were determined by comparison with a 100 bp ladder, and are listed as number of base pairs per observable band

(+) indicates presence of that size band; blank cells indicate no band of that size was detected

Table 6 Marker sensitivity screening

Markers	Bók Turkey (Female Parent)	Shuqualak (Male Parent)	Progeny 1	Progeny 2
RM251	305	311	305	305
RM259	147	109, 261	109, 147	109, 261
OPA-08	133	121	133	133
MCSA176C03	160, 174	160, 174	160, 174	160, 174
YCS02.047	126, 151	126, 153	126, 151	126, 151

A. gigantea specimens growing > 300 km apart were transplanted and manually crossed
Parents and progeny were screened with five markers determined polymorphic using the ABI 3130xl
Numbers indicate each band size in base pairs; a single number indicates homozygosity at that locus

Table 7 Total alleles detected in Mississippi and North Carolina rivercane populations

Alleles detected in North Carolina Rivercane				Alleles detected in Mississippi Rivercane	
RM251 (57)		RM259 (33)		RM251 (2)	RM259 (6)
101	157	109	330	305	130
102	158	112	351	311	136
104	163	113	375		210
105	164	130	379		211
106	165	131	404		261
108	167	135			262
109	168	136			
110	175	137			
111	184	140			
112	193	141			
114	203	143			
117	228	145			
119	229	146			
120	251	147			
121	257	155			
122	258	163			
123	259	187			
124	260	190			
125	266	193			
126	273	202			
130	276	204			
133	284	264			
135	291	265			
136	305	275			
147	310	288			
151	311	289			
153	328	293			
154	360	296			
156					

Numbers indicate each band size in base pairs

Table 8 Estimated heterozygosity and F-statistics of Mississippi canebrakes

Canebrake		*N*	*Na*	*Ne*	*Ho*	*He*	*F*
Bók Turkey	Mean	7.000	2.000	1.587	0.200	0.270	0.259
	SE	2.000	1.000	0.587	0.200	0.270	0.300
Cane Trail	Mean	4.500	2.000	1.567	0.125	0.266	0.529
	SE	0.500	1.000	0.567	0.125	0.266	0.304
Culvert	Mean	40.000	1.500	1.284	0.188	0.181	-0.035
	SE	0.000	0.500	0.284	0.188	0.181	0.183
Dahomey	Mean	13.500	2.000	1.480	0.309	0.283	-0.081
	SE	3.500	0.000	0.355	0.191	0.172	0.018
Shuqualak	Mean	14.500	2.500	1.619	0.393	0.324	0.249
	SE	0.500	0.500	0.477	0.393	0.199	0.751
Stallo	Mean	10.000	2.000	1.580	0.111	0.269	0.586
	SE	1.000	1.000	0.580	0.111	0.269	0.284

Table 8 (Continued)

F-Statistics	F_{ST}
Mean	0.523
SE	0.389

Alleles detected were analyzed statistically using methods found in the statistics section of materials and methods; markers used were RM251 and RM259
N= number of samples, *Na*=number of alleles (arithmetic mean), *Ne*=number of effective alleles, *Ho*= observed heterozygosity, *He*=expected heterozygosity, *F*=Wright's fixation index, SE=sample error (error calculated by observing a sample instead of a whole population); for $F_{ST,}$ refer to materials and methods

Table 9 Analysis of molecular variance (AMOVA) within and among Mississippi rivercane brakes

Source	DF	SS	MS	Est. Var.	Variance (%)
Among Canebrakes	5	95.119	19.024	1.049	46
Within Canebrakes	104	125.808	1.210	1.210	54
Total	109	220.927		2.258	100
Statistic	Value	*p* value			
ΦPT	0.464	$p<0.001$			

Alleles detected were analyzed statistically using methods found in the statistics section of materials and methods; alleles were input into a genetic distance matrix which was used to measure variation statistically, based on the allele size in base pairs (see Tables 7, 8); markers used were RM251 and RM259
DF=degrees of freedom, SS=sum of squares, MS=mean squares, Est.Var.=estimated variance, *ΦPT*=population pairwise value, *p*=probability observed values are greater than expected by chance (checking the random mating hypothesis)

Table 10 Estimated heterozygosity and F-statistics of North Carolina canebrakes

Canebrake		*N*	*Na*	*Ne*	*Ho*	*He*	*F*
Cherokee	Mean	64.500	27.000	6.424	0.375	0.840	0.562
	SE	29.500	11.000	1.074	0.232	0.027	0.262
Cullowhee	Mean	18.000	13.000	6.446	0.613	0.833	0.259
	SE	11.000	3.000	1.721	0.042	0.045	0.090

F-Statistics-All Tissues	F_{ST}
Mean	0.042
SE	0.035
F-statistics Maternal Tissue only	
Mean	0.054
SE	0.030

Alleles detected were analyzed statistically using methods found in the statistics section of materials and methods; markers used were RM251 and RM259
N= number of samples, *Na*=number of alleles (arithmetic mean), *Ne*=number of effective alleles, *Ho*= observed heterozygosity, *He*=expected heterozygosity, *F*=Wright's fixation index, SE=sample error (error calculated by observing a sample instead of a whole population); for $F_{ST,}$ refer to materials and methods

Table 11 Analysis of molecular variance (AMOVA) within and among North Carolina rivercane brakes

AMOVA-All Tissues					
Source	DF	SS	MS	Est. Var.	Variance (%)
Among Canebrakes	1	3.178	3.178	0.021	1
Within Canebrakes	129	276.471	2.143	2.143	99
Total	130	279.649		2.165	100
Statistic	Value	*p* value			
ΦPT	0.010	0.117			
AMOVA- Maternal Tissue Only					
Source	DF	SS	MS	Est. Var.	Variance (%)
Among Canebrakes	1	0.752	0.752	0.000	0
Within Canebrakes	62	86.358	1.393	1.393	100
Total	63	87.109		1.393	100
Statistic	Value	*p* value			
ΦPT	-0.020	0.914			

Alleles detected were analyzed statistically using methods found in the statistics section of materials and methods; alleles were input into a genetic distance matrix which was used to measure variation statistically, based on the allele size in base pairs (see Tables 7, 10); markers used were RM251 and RM259

DF=degrees of freedom, SS=sum of squares, MS=mean squares, Est.Var.=estimated variance, *ΦPT*=population pairwise value, *p*=probability observed values are greater than expected by chance (checking the random mating hypothesis)

Table 12 Pairwise population matrix of Nei's genetic distance (*D*) and genetic identity (*I*) of Mississippi canebrakes

Nei's (*D*)						
Bók Turkey	Cane Trail	Culvert	Dahomey	Shuqualak	Stallo	
0.000						Bók Turkey
0.148	0.000					Cane Trail
0.230	0.049	0.000				Culvert
0.072	0.042	0.065	0.000			Dahomey
1.157	1.064	1.033	0.802	0.000		Shuqualak
1.296	1.309	1.296	0.957	0.005	0.000	Stallo
Nei's (*I*)						
Bók Turkey	Cane Trail	Culvert	Dahomey	Shuqualak	Stallo	
1.000						Bók Turkey
0.862	1.000					Cane Trail
0.795	0.952	1.000				Culvert
0.930	0.959	0.937	1.000			Dahomey
0.314	0.345	0.356	0.449	1.000		Shuqualak
0.274	0.270	0.274	0.384	0.995	1.000	Stallo

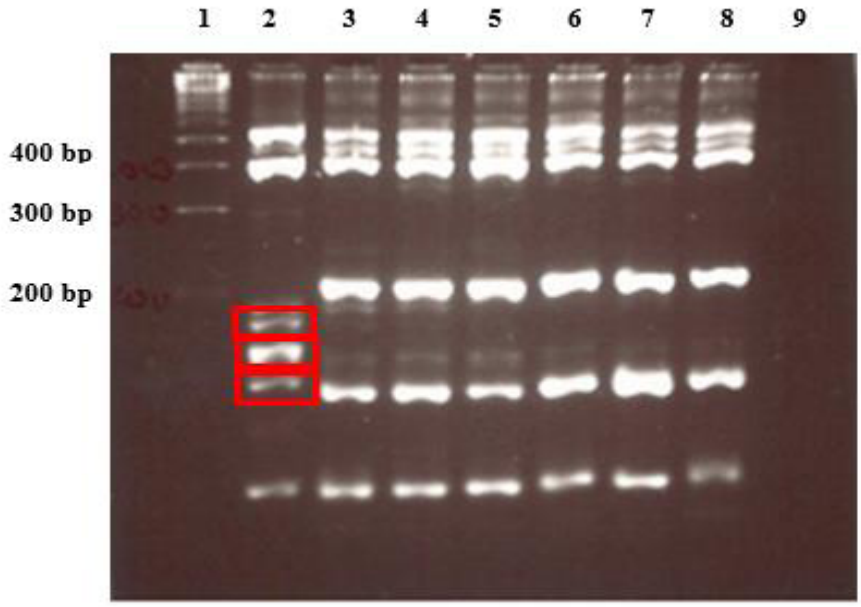

Figure 3 Sugarcane marker MCSA176C03

Agarose gel electrophoresis of PCR-amplified plant DNA. Gel contained DNA ladder (lane 1), *A. tecta* (lane 2), Dahomey siblings (lanes 3-5), Uski Chitto (lane 6), Bók Shankolo (lane 7), Gum Branch (lane 8) and negative control (lane 9). Negative control was master mix sans DNA. Boxes indicate polymorphism between species *A.tecta* (lane 2) and *A. gigantea* lanes 3-8.

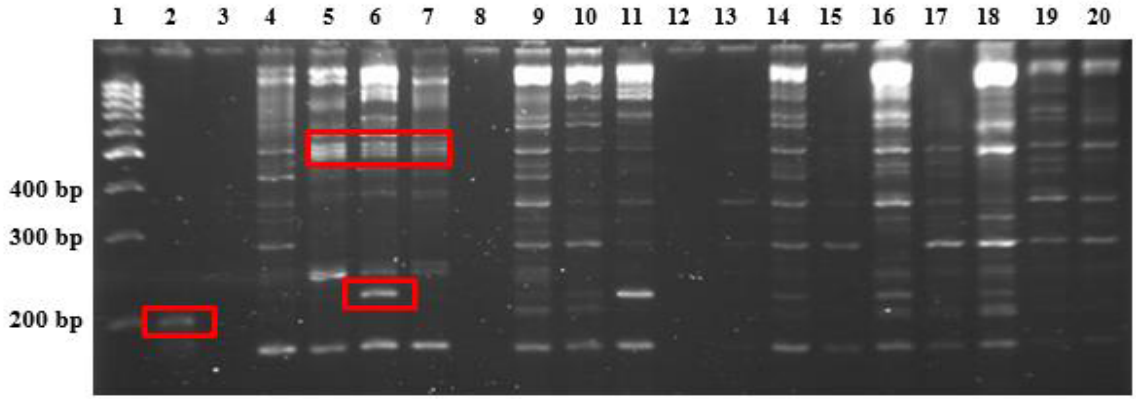

Figure 4 Sugarcane marker MCSA180E02

Agarose gel electrophoresis of PCR-amplified plant DNA. Gel contained DNA ladder (lane 1), *Phyllostachys nigra* (lane 2) and *P. aurea* (lane 3) used as controls for species sensitivity, and *A. tecta* (lane 5=leaves, lane 6= leaves, lane 7=shoots). Lanes 4 and 8-20 contained rivercane samples from the Dahomey site and are listed in the order on the gel (all were Dahomey samples): 2, 17, 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, and 14. Boxes indicate polymorphism between species, *Phyllostachys nigra* (lane 2), *A. tecta*, (lanes 3-5).

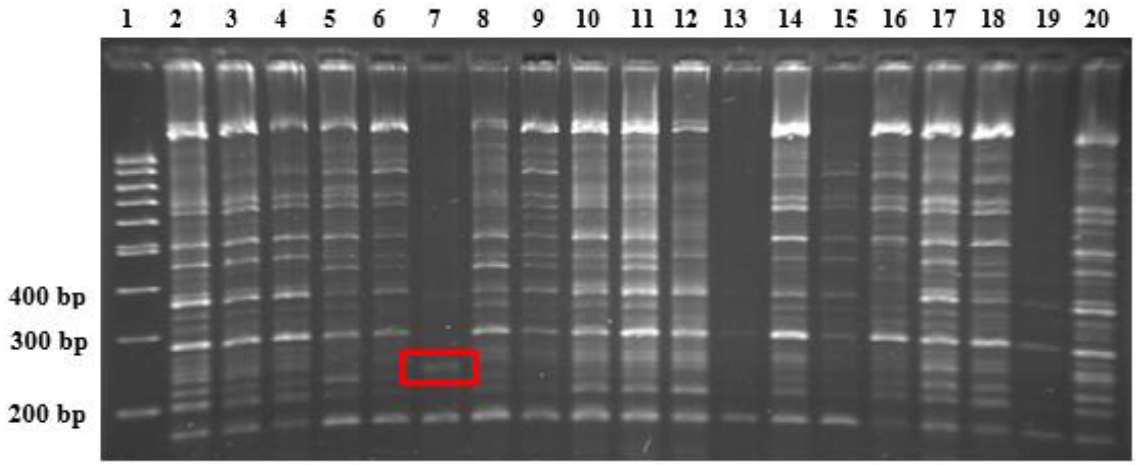

Figure 5 Sugarcane marker MCSA180E02

Agarose gel electrophoresis of plant DNA. Gel contained DNA ladder (lane 1), *A. gigantea* samples from across the state of Mississippi (lanes 2-6, 8-19) presented in order: Dahomey 15, Dahomey 16, Dahomey18, Uski Chitto, Oktoc, Gum Branch, Nanih Waiya, Cooonewah, Choctaw Lake, Skuna Loosa, Chinchahoma, Tusca Animpulli, Coffeeville PMC, Oktibbeha, Tibbee II N, Tillatoba, and Bók Chulaffi Kapassachi. Remainder included *Pseudosasa japonica* (lane 7, also called Pseudo Oktoc in Table 1), and Moss Island 3 (Tennessee rivercane, lane 20). Box indicates polymorphism and lack of bands between *Pseudosasa japonica* (lane 7) and *A. gigantea*

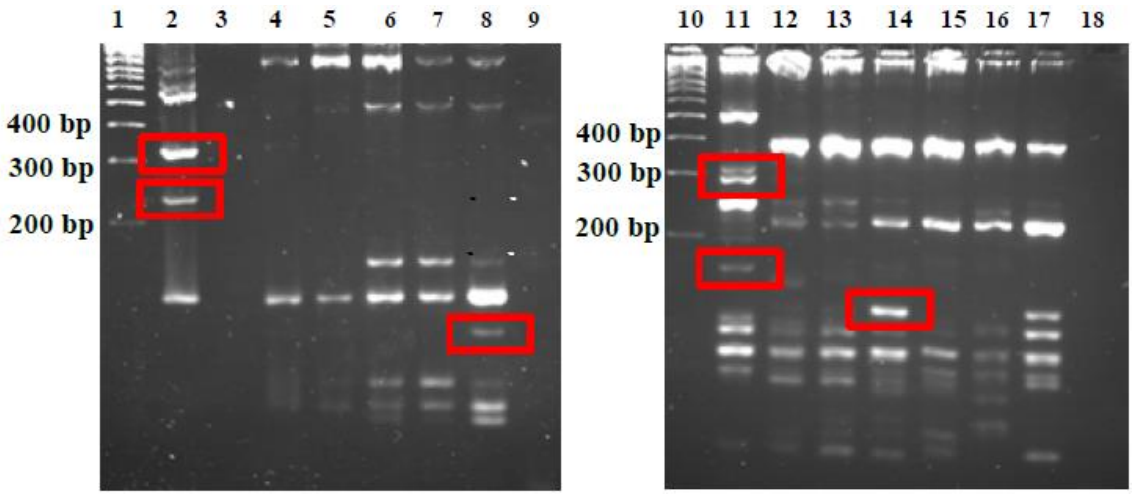

Figure 6 Sugarcane markers MCSA062B06 and MCSA175G03

Agarose gel electrophoresis of PCR-amplified plant DNA. DNA in lanes 2-9 were amplified using marker MCSA062B06; lanes 11-18 used marker MCSA175G03. Gels contained DNA ladder (lanes 1, 10), *A. tecta* (lanes 2, 11), lane 3-empty by error, Dahomey siblings (lanes 4-5, 12-14), Uski Chitto (lanes 6, 15), Bók Shankolo (lanes 7, 16), Gum Branch (lanes 8, 17), and negative control (lanes 9, 18). Negative control was master mix sans DNA. Boxes indicate polymorphism between *A. tecta* (lanes 2 and 11) and *A. giganatea* and a couple of polymorphic band examples within *A.gigantea* samples (lane 8 and 14).

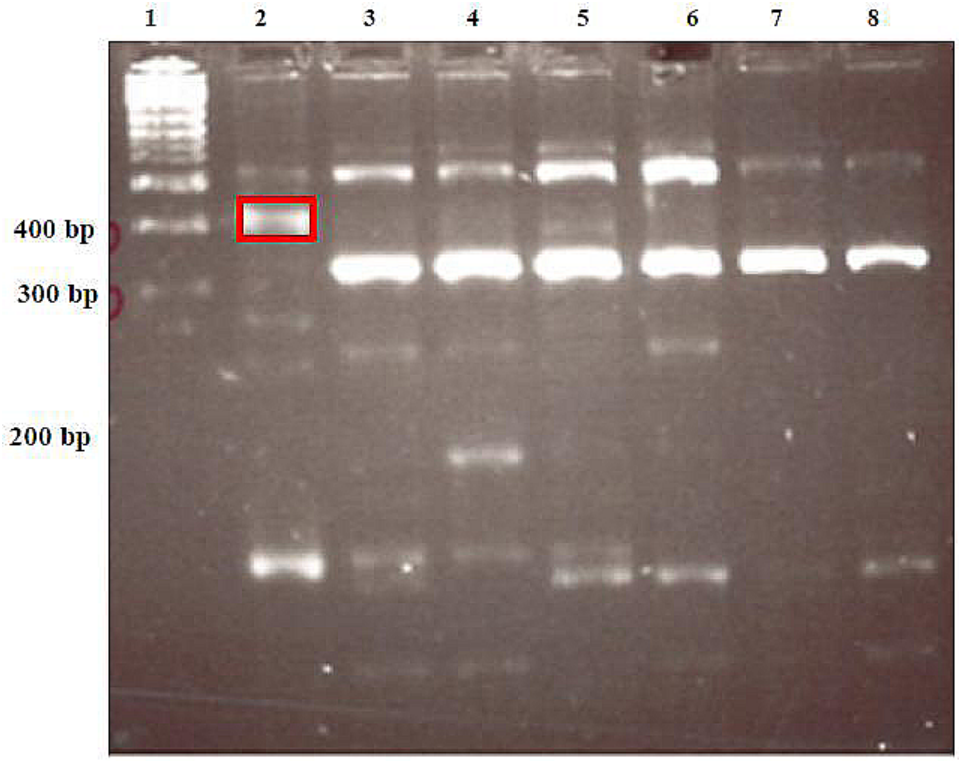

Figure 7 Rice marker RM30

Agarose gel electrophoresis of PCR-amplified plant DNA. Gel contained DNA ladder (lane 1), *A. tecta* (lane 2), Dahomey siblings (lanes 3-5), Uski Chitto (lane 6), Bók Shankolo (lane 7), and Gum Branch (lane 8).
200 bp marker not visible because of picture quality, so its approximate location is indicated

CHAPTER IV

DISCUSSION

Arundinaria gigantea (rivercane) is one of three bamboo species native to North America. It can reproduce both vegetatively through rhizomes and sexually through cross-pollination. Historically, rivercane brakes were found throughout the southeastern U.S. (Stewart 2007), but were greatly depleted due to agricultural applications and urbanization (Platt et al. 2009), so much so to be listed as an endangered ecosystem (Noss et al. 1995). Rivercane plays integral roles in providing habitats for wildlife and raw materials for traditional Native American basketry, and can act as a riparian buffer, to name a few.

It is believed that the fragmentation of canebrakes has led to reduced fecundity due to brakes being composed of genetically-similar clones, thereby limiting the ability to regenerate the brakes through seed/seedling generation. Development and assessment of molecular markers to determine genetic relatedness of rivercane plants within and between brakes would assist in this determination. In addition, markers that could distinguish between related plants, such as siblings, would ensure the preciseness of these markers, important for use in restoration efforts. This type of molecular marker analysis and genetic fingerprinting has been conducted using amplified fragment length polymorphisms (AFLP) to distinguish rivercane from *A. tecta* and *A. appalachiana* as well as other temperate bamboo species (Triplett and Clark 2010, Triplett et al. 2010).

This current study used more precise markers, simple sequence repeat (SSR) and sequence-characterized amplified region (SCAR), and focused on assessing rivercane genotypes among and between canebrakes.

Numerous samples (352) of *A. gigantea* were processed for DNA isolation and extraction. Initially, 22 markers (10 SSR, 2 SCAR) were evaluated by agarose gel electrophoresis (Table 2); four were unable to be successfully amplified using rivercane DNA. Of the 10 initial *O. sativa* markers (Chen et al. 1997) analyzed, seven were assessed with four determined to be polymorphic within *A. gigantea*; of the 10 initial *Saccharum* spp. markers (Sharma et al. 2008) analyzed, nine were assessed with four determined to be polymorphic within *A. gigantea*. Both *Bambusa* spp. markers (PW-02, OPA-08; Das et al. 2005) were polymorphic in *A. gigantea*. Therefore, 56% (10/18) of the markers assessed were determined to be polymorphic between rivercane samples using agarose gel electrophoresis (Table 4).

During optimization and standardization of amplification conditions using these markers/primers, only Mississippi rivercane, Tennessee rivercane and control bamboo species were used (they were the samples that had been isolated and processed at that time), so this might have contributed to the inability to optimize amplification conditions for four of the above-mentioned 22 markers. After optimizations, the plant panels assessed via agarose gel electrophoresis generally consisted of samples of known siblings (half-siblings, Dahomey), samples of additional Mississippi rivercane, and other members of Bambusoideae (Figures 3-6) to show sensitivity of the markers being assessed within and between genera and between *Arundinaria* species. *A. tecta* displayed distinct genetic profiles compared to rivercane using sugarcane markers (Figures 3, 6),

which corroborated results of Triplett et al. (2010) which concluded that *A. tecta* is indeed a separate sister species of *A. gigantea*. The approach delineated in this thesis was similar to that of Nayak et al. (2003) where RAPD markers were used to investigate genetic bamboo phylogeny. Not all genera could be successfully analyzed using these 18 markers; different genera either yielded distinct bands in their genetic profile or did not amplify as well such as marker MCSA180E02 (Figures 4, 5). Lack of amplification could relate to PCR conditions that were primarily optimized using rivercane. With the primary focus on rivercane, an example of a marker that could distinguish numerous rivercane samples via agarose gel electrophoresis was RM30 (Table 5).

Use of the ABI 3130xl enabled more precise determination of genetic diversity and differentiation of rivercane plants using fluorescently-labeled markers. The focus was on primers proven to be polymorphic via gel analysis. This sensitive system better-enabled detection of polymorphisms in and among non-seed producing and seed-producing canebrakes. This type of analysis was particularly important in determining genetic differences among members of the same canebrake and among known siblings. In fact, one marker (RM251) yielded a total of 57 alleles and RM259 yielded a total of 37 alleles (29 unique to this marker) using the ABI 3130xl (Table 7).

A canebrake that was assessed in detail was Dahomey. Gel and capillary analysis confirmed genetic variation (Figures 4-6, Table 8). In fact, this canebrake displayed the greatest polymorphism compared to the five other Mississippi canebrakes assessed based on F ($F = -0.081$; Table 8). According to the statistical program used, GenAlEx (Peakall and Smouse 2006, 2012), an F index < 0 indicates excess genetic variation or the intentional selection of heterozygotes during sampling. However, recall that this

canebrake was composed of siblings (half-siblings), so these results were expected. Culvert also had a negative F value (F = -0.035; Table 8), indicating genetic diversity within this canebrake. In regard to the F value calculated for North Carolina canebrakes, both values were greater than zero (Table 10), which is opposite than expected since these canebrakes are fertile. This increased homozygosity was noted but the F value statistic focuses almost entirely on the heterzygosity of the loci tested. A high F value could mean homozygosity at those loci but the overall genetic diversity of a population or canbebrake (at those same loci) could however still be significantly high.

At least one of the molecular markers assessed (RM259) could also distinguish between two full siblings, indicating the sensitivity of this marker for determination of genetic relatedness (Table 6). The four other markers tested were not as sensitive, although could be used to discern more genetically distinct individuals. Overall, the confirmation of markers identified by Chen et al. (1997), Cordeiro et al. (2001), Das et al. (2005), and Sharma et al. (2008) will be of great utility for future genetic studies in rivercane. Identifying additional markers would enhance these efforts.

In further genetic analyses of rivercane canebrakes in both Mississippi and North Carolina, markers RM251 and RM259 were used because of their ability to distinguish rivercane genotypes and their reproducibility using capillary electrophoresis. At least three markers should have been selected for use with the ABI 3130xl system to reduce potentially skewing results . The use of three or more markers would confirm validity of these results in future studies. Sample size was also an issue. However, by using codominant SSR markers, we were able to compensate for limited sample sizes of

Mississippi canebrakes to gain an accurate estimate of their genetic diversity using GenAlEx (Peakall and Smouse 2006, 2012).

In regard to relatedness of individuals among canebrakes, Cane Trail, Culvert and Dahomey showed a great degree of genetic relatedness (low Nei's *D* values for genetic distance, high Nei's *I* values for genetic identity; Table 12). Due to their short geographical distances (Figures 1, 2) and this noted genetic relatedness, they could have been part of the same canebrake, historically. Currently, these canebrakes are separated by a highway and cultivated/farming areas. The Cane Trail canebrake was 1500 m away from Culvert and 1500 m from Dahomey (Figure 2). Cane Trail displayed 95-96% genetic identity (relatedness) to each of these canebrakes (Table 12). Although Culvert and Dahomey brakes were nearly twice that distance, they displayed Nei's *I* of 94%. These three canebrakes did not display high genetic identities with any of the other three Mississippi canebrakes, thereby reinforcing the hypothesis that they had been part of the same canebrake.

The fixation index (F_{ST}) observed among the Mississippi canebrakes was 0.523 (Table 8); the range of values for this parameter is 0-1.0, with 1.0 indicating total genetic differentiation. The population pairwise value (ΦPT) of 0.464 (Table 9) is close to that value. It is estimated that there is 46% genetic diversity among canebrakes and 54% within canebrakes. Both F_{ST} and ΦPT values suggest genetic bottlenecks (little genetic diversity) within canebrakes caused by historic fragmentation of canebrakes (Stewart 2007), limiting genetic diversity.

In contrast, the North Carolina canebrakes exhibited a F_{ST} of 0.042 (all tissues) which was consistent with the ΦPT value of 0.010 indicating there is 1% genetic

diversity among canebrakes and 99% within canebrakes (Tables 10, 11). These values were expected since both NC canebrakes were capable of viable seed production. Due to analysis of just two canebrakes, these values may be less accurate than those for the Mississippi canebrakes. However, since the NC and MS values are so different, conclusions should still be able to be drawn. Data suggests that the North Carolina canebrakes are successfully undergoing random mating (cross-pollination) as evidenced by the abundance of viable seed produced after flowering events.

In these genetic analyses, determinations of genetic diversity are important in assessing each canebrake. Equally important, if not more so, would be to determine how many genetically diverse individuals would be needed to ensure a healthy canebrake capable of viable seed generation. According to Wright (1945, 1951, 1965) the number of effective alleles (*Ne*) represent distinct individuals in a population. Comparing this value for the -less fertile Mississippi canebrakes with the fertile North Carolina canebrakes, the number of distinct genotypes needed for a healthy canebrake could be estimated. As expected, the *Ne* values for the NC canebrakes were much higher than those for the MS canebrakes. The NC values of 6.424 and 6.446 (Table 10) suggest that at least seven distinct individuals are required to enable random mating and production of viable seeds in rivercane brakes.

In contrast, the *Ne* values for the Mississippi canebrakes ranged from 1.284 to 1.619 (Table 8), indicating limited genetic diversity and lack of the critical number of distinct individuals to maintain a healthy canebrake. It is possible that during sampling, multiple samples of the same genet could have been collected due to vegetative propagation and ability of bamboo rhizomes to spread great distances (Suyama et al.

2000), thereby contributing to lower *Ne* values. This can be an issue since Suyama et al. (2000) confirmed that a single clone could spread across a distance of 300 m. The Culvert canebrake was measured to span 814 m in length (Figure 2). Only two effective alleles (*Ne*) were found in this entire canebrake (Table 8). So, theoretically, two clones might have dominated and spread through this brake, thereby reducing the probability that other potential genotypes would be randomly sampled. If this was the case, then values for the Mississippi canebrakes might have over-estimated the clonal nature of these canebrakes. A good control that negates this is the Dahomey canebrake that was composed of seedlings from a flowering event. It displayed similar values (*Ne* and others) to geographically-close canebrakes (Cane Trail and Culvert) and values were within similar ranges for all parameters assessed in the six MS canebrakes (Table 8).

In regard to Dahomey, even with a low *Ne* value (1.480; Table 8), it was capable of a flowering event, albeit quite limited. One floret produced viable seeds, so cross-pollination was proven successful in this canebrake, and these Dahomey siblings were determined to contain greater genetic diversity than any other Mississippi "canebrake" in analyses discussed previously. This should be considered a successful flowering event, but probably not a healthy canebrake because it is unknown whether seed-generation can be maintained in this canebrake in the future. Also, as previously mentioned, the Dahomey canebrake was a collection of seeds and the progeny of a single flowering ramet.

For successful establishment of a stand of rivercane and other outbred temperate bamboos, several genetically distinct genotypes are required to ensure long-term viability. A beneficial future study could include a more precise determination of how

many genotypes would be needed to create a healthy canebrake. Additional assessments of the number and relatedness of the different genotypes in fertile North Carolina canebrakes could assist in this effort.

Additional studies could also assist in better-understanding flowering events and identification of molecular markers (such as for sexual compatibility/incompatibility) that could be tracked to ensure successful cross-pollinations. In general, more information about the genetic makeup of rivercane as well as other temperate bamboos would assist. In fact, sequencing the *A. gigantea* genome would be a gigantic step toward better-understanding and conserving this species.

Overall, this study was conducted with three goals in mind: (1) to develop genotyping methods for *A. gigantea* to begin to determine and assess genetic characteristics, (2) to assess and characterize existing canebrakes (canebrakes) for genetic diversity and differentiation, and (3) to use this knowledge to assist in generation of healthy canebrakes. Genetic analyses in this study support self-incompatibility due to the need for cross-pollination for viable seed production. This, along with rivercane's ability to spread, clonally, over vast distances and bamboo's sporadic flowering (once per 30-40 years; Judziewicz et al. 1999) facilitate clones being established; when the flowering event does occur, the entire brake can collapse due to no viable seeds being generated due to the clonal nature of the brake. These are daunting challenges to restoring and maintaining Mississippi's endangered habitat.

The first step in addressing these challenges has been the genetic analysis of *A. gigantea*. This study has laid the groundwork to determine and assess genetic relatedness of discrete rivercane plants and canebrakes which can be applied to restore existing

brakes and generate new brakes. One simple step toward creating healthy canebrakes would be to harvest and maintain rivercane clones that represent the different genotypes assessed in this study. They could be used in the future to create healthy synthetic canebrakes.

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